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Factors in the environment, viruses and host responses affecting the epidemiology of tick-borne encephalitis virus in Northern Europe

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Bank vole in the cover by Jonna Koski

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To my family including the big glad one

Abstract

Tick-borne encephalitis virus (TBEV) circulates mainly in the *Ixodes ricinus* and *Ixodes persulcatus* tick species, which serve both as hosts and vectors for the virus. Wild rodents are considered as bridges for non-viremic transmission between the ticks, which is the most important maintenance factor for TBEV. Secondary hosts support TBEV circulation as bloodmeal sources for ticks. The fragile maintenance cycle of the virus is affected by climates and availability of biotic factors thus, TBEV is found only in restricted foci by the Baltic Sea and the biggest lakes in Finland.

TBEV is transmitted to humans when bitten by an infected tick. Infection may lead to a clinical disease, tick-borne encephalitis (TBE). The number of TBE cases has increased in Europe since the 1980s. Disease manifestation ranges from mild flu-like illness to inflammation of the central nervous system (CNS), and is in some cases followed by severe quality-of-life impairing sequelae. The clinical picture varies according to the virus subtype, as well as the age and genetic background of the patient.

Three subtypes of TBEV are known: European (TBEV-Eur), Siberia (TBEV-Sib) and Far-Eastern (TBEV-FE). TBEV-Eur is carried mainly by *I. ricinus* and the two latter subtypes by *I. persulcatus*. Finland lies in the mixing zone of the tick species as both of the main host tick species and two of the three TBEV subtypes, TBEV-Eur and TBEV-Sib, are endemic in Finland. The disease has been known in Åland islands since the 1950s as Kumlinge disease. Also the south-western archipelago and the Lappeenranta region by Lake Saimaa waterway in South-Eastern Finland have been known to be endemic for decades.

TBE is a notifiable disease in Finland. All laboratory diagnosed cases are reported to the Institute of Health and Welfare by the treating hospital district, often the one of the municipality of residence of the patient. In the present study we surveyed all human cases reported in Finland during 2007-2013 by the geographical place of infection. We also surveyed the diagnostic alertness for TBE in different hospital districts in Finland and among patients with neurological infections with unknown aetiology. The number of suspected patients with TBE doubled during the period of our survey from 563 to 1154. However, TBE was not significantly underdiagnosed among patients with neurological infections with unknown aetiology.

Besides the previously known endemic areas, infections were reported in a wider region around Saimaa, in central Finland and at the coast of Gulf of Finland. The areas with repeated human TBE cases were found in the northern part of the west coast, north of 64° latitude.

We studied further in more detail several geographical sites of human infections. In Simo, Finnish Lapland the major tick species was *I. persulcatus,* as is expected in the north, but it unexpectedly carried the TBEV-Eur subtype. Therefore, we suggest that tick species in the area is not preventative for establishment of any newly introduced TBEV-subtype.

Also, the species distribution of small mammals at the geographical sites of human infections was studied. While *Apodemus* mice are considered the most important hosts for TBEV maintenance in the deciduous zone, the bank vole, *Myodes glareolus* was the dominant species in the sites studied in Finland. An exception was the island Isosaari in Helsinki, where the vole species was exclusively *Microtus agrestis*, the field vole.

To further study the infection kinetics and persistence of TBEV in the natural host species in the boreal zone as well as to compare the TBEV subtypes, we infected colonized bank voles with strains representing each of the three known TBEV subtypes.

All strains were infective and highly neurotropic. TBEV-RNA could be detected in the brain as long as 168 days post infection. Clearance of TBEV-RNA from the brain was significantly slower than from the other organs investigated. However, attempts to show infectivity in cell culture were not successful. TBEV-FE induced prolonged viremia, indicating that its kinetics in rodents may differ from that of the other two subtypes. Altogether, the study showed that bank voles can develop TBEV infection of the CNS with inflammation and other pathological findings comparable with encephalitis. However, clinical symptoms were seen only in a few individuals and thus bank voles can serve as resistant models for studies on tick-borne encephalitis. Persistence of viral RNA in the brain of animals with asymptomatic course of infection supported our findings in wild rodents: TBEV-RNA was detectable in the brain of bank voles and field voles in winter several months after tick-feeding season in both TBEV-Sib and TBEV-Eur endemic areas (Kokkola and Isosaari, respectively). It is unlikely, that the individuals would have survived until February and March having manifested symptoms or impaired functional abilities.

Serological analyses on wild rodents and laboratory animals support the suggestion that rodents may serve as sentinels for TBEV endemicity. However, the detection method, target organ, trapping season, sentinel species, and ecological parameters of the trapping site should be considered carefully when interpreting the results.

List of original publications

I: **Tonteri E,** Jääskeläinen AE, Tikkakoski T, Voutilainen L, Niemimaa J, Henttonen H, Vaheri A, Vapalahti O. Tick-borne encephalitis virus in wild rodents in winter, Finland, 2008-2009. Emerg Infect Dis. 2011;17(1):72-5.

II: Jääskeläinen AE, **Tonteri E,** Sironen T, Pakarinen L, Vaheri A, Vapalahti O. European subtype tick-borne encephalitis virus in Ixodes persulcatus ticks. Emerg Infect Dis. 2011;17(2):323-5.

III: **Tonteri E**, Kipar A, Voutilainen L, Vene S, Vaheri A, Vapalahti O, Lundkvist Å. The three subtypes of tick-borne encephalitis virus induce encephalitis in a natural host, the bank vole (Myodes glareolus). PLoS ONE, 2013, Vol 8(12), pp e81214 2013;8(12):e81214.

IV: **Tonteri E**, Kurkela S, Timonen S, Manni T, Vuorinen T, Kuusi M, Vapalahti O. Surveillance of endemic foci of tick-borne encephalitis in Finland -Evidence of emergence of new foci. Manuscript in preparation

Table of Contents

List of abbreviations

Arboviruses	viruses transmitted by arthropod vector
BBB	blood-brain barrier
BSA	bovine serum albumin
BSL-3	biosafety level 3
C	capsid (protein)
CEE	Central European early summer encephalitis
CNS	central nervous system
CSF	cerebrospinal fluid
CT-value	cycle threshold value
Dpi	days post infection
E	envelope (protein)
ECDC	European Centre for Disease Prevention and Control
EEG	electroencephalogram
EIA	enzyme immuno assay
FCS	fetal calf serum
FSME	frühsommer-meningoenzephalitis
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HUS	Hospital District of Helsinki and Uusimaa
IFA	immunofluorecence assay
Ig	immunoglobulin
JEV	Japanese encephalitis virus
LIV	louping ill virus
M	membrane (protein)
MEM	minimum essential medium
MRI	magnetic resonance imaging
Nm	nano metres
NS	non structural
ORF	open reading frame
PBS	phosphate-buffered Saline
prM	precursor membrane protein
PCR	polymerase chain reaction
RNA	ribonucleic acid
RSSE	Russian early summer encephalitis
SPECT	single-photon-emission computed tomography
TBE	tick-borne encephalitis
TBEV	tick-borne encephalitis virus
TBEV-Eur	tick-borne encephalitis virus, European subtype
TBEV-FE	tick-borne encephalitis virus, Far-Eastern subtype
TBEV-Sib	tick-borne encephalitis virus, Siberian subtype
WNV	West Nile virus
YFV	yellow fever virus
µl	micro litre

Review of the literature

Introduction

The tick-borne encephalitis viruses are a group of tick-borne flaviviruses, commonly called with a singular form tick-borne encephalitis virus, TBEV, that are maintained in nature in a complex cycle determined by *Ixodes* ticks and their host vertebrates. Tick-borne encephalitis virus is a causative agent of the human disease, tick-borne encephalitis (TBE). The infection is transmitted via bite from an infected tick or via the alimentary route, when consuming contaminated unpasteurized milk of ruminants [1, 2]. TBE infection may occur asymptomatically or manifest as a mild flu-like illness or with more severe features such as meningitis or severe encephalitis with subsequent sequelae [3]. The case numbers in Europe and Russia combined totalled 5352 in 2008, having declined from 12733 during the peak years of 90's [4]. In Finland, TBEV is not as big a concern (20-40 cases/year) as in the neighbouring countries Sweden (100-200 cases/year) or Estonia (100-250 cases/year) [5] or in the most important endemic country, Russia.

Zoonotic diseases: a short introduction

By definition, emerging infection is an infection for which the incidence has increased rapidly, typically in certain geographical range. The pathogen may be novel or re-emerging [6]. Of the emerging infectious diseases 60,3 to 70% are zoonotic, i.e. diseases that are transmitted between animals and humans, the majority of them being viruses arising from wildlife [7-9]. Indeed, very few infectious diseases are restricted to humans. A zoonotic pathogen may be transmitted directly from a reservoir host to humans, indirectly via contaminated food or water, or it may circulate in a cycle involving the animal host, arthropod vector (sometimes acting also as reservoir host) and humans [8, 10, 11]. Humans often serve only as spill-over dead-end hosts for pathogens circulating in wildlife or/and domestic animals i.e. the infection is self-limiting in humans thus the infection advances the maintenance of the virus only if a human derived viruses infect a maintenance host of the virus. However adaptation to human-to-human transmitted infection may occur. Even if a human population would gradually gain immunity to the pathogen, naturally or by vaccinations, animal reservoirs enables re-emergence of the pathogen in naïve human populations. The pathogen may also mutate while circulating in the reservoir, thus eradication is challenging and even impossible [10].

The fast expansion of the human population has led to urbanization and significant changes in land use, which provides more contacts with wildlife and new habitats for arthropod vectors. The development has also even reduced the biodiversity [12-14]. Travelling and trade accelerate rapid introduction of new vector species and pathogens in previously naïve areas with severe consequences, as has been observed in history several times. Not only is there direct impact on human morbidity, but also diseases of domestic animals and wildlife may affect local communities [14, 15]. Climate change has been predicted to lead further to more dramatic changes in land use and human mass movements with expansion of endemic areas of some

arthropod vector species, thereby contributing to the emergence and re-emergence of zoonotic pathogens [16]. On the other hand, some pathogens may disappear.

The family *Flaviviridae*, genus *Flavivirus*

The family *Flaviviridae* includes several viruses pathogenic to humans, domestic animals and wildlife species [17]. The genus *Flavivirus* (referred to in this thesis as flaviviruses) contains 53 species [18]. They differ from the other members of the *Flaviviridae* family as flaviviruses are, apart from a few species, arthropod-borne viruses (arboviruses) and many of them infect both vertebrate and hematophagous invertebrate species. Host-vector dynamics largely determine the evolution and the ecology of flaviviruses [17, 19, 20].

Flaviviruses that infect vertebrates are categorized according to their major arthropod vector species and on serological basis as mosquito-borne viruses and tick-borne viruses. Viruses without a known arthropod vector are assumed to infect only vertebrates. These include two species of *Pestivirus* (pestis=plague), *Hepacivirus* (hepatos=liver) and two species of *Pegivirus* (*persistent G-virus*) [20-25]. In addition, a tentative group of flaviviruses is specific to insects alone [24].

Mosquito-borne flaviviruses

Mosquitos are the most important and most common arthropod host among arboviruses [26]. They serve as vectors for viruses responsible for a significant share of the global infectious disease burden in humans such as Dengue viruses (serotypes 1-4) yellow fever (YFV), West Nile (WNV), Japanese encephalitis (JEV), Murray valley encephalitis, Rocio and St Louis encephalitis viruses [19, 21-23, 27, 28]. Two clades of mosquito-borne flaviviruses, *Culex* and *Aedes*, can be distinguished by the vector species and further by pathological characteristics, although the latter are more heterogenous [23]. The *Aedes* group also includes viruses found in Northern Europe such as Lammi and Hanko viruses [29, 30]. Dengue, West Nile and yellow fever viruses are an exception among flaviviruses as they can be transmitted between people by mosquitos, while for other flaviviruses humans serve only as accidental dead-end hosts (incompetent host) [19].

Tick-borne Flaviviruses

The 12 species in the group of tick-borne flaviviruses have been divided further into three subgroups: the sea-bird tick-borne viruses, the mammalian tick-borne viruses and the recently added Kadam virus group [18, 20].

The mammalian tick-borne flavivirus group, forming also the tick-borne encephalitis serogroup [31, 32], includes several zoonotic human pathogens, which manifest as mild to moderate febrile illnesses or severe encephalitis and haemorrhagic disease [19, 33] (figure 1). In addition to humans, several other mammalian species have been found to develop clinical illness [32]. Even if the geographical distribution of each individual tick-borne flavivirus species, except for TBEV, is restricted, the members of the group are found widely in Asia, Africa,

Australia, Europe and North America. Many of them together with so far unknown tick-borne flaviviruses have the potential to emerge as new or altered human or animal pathogens [19, 32].

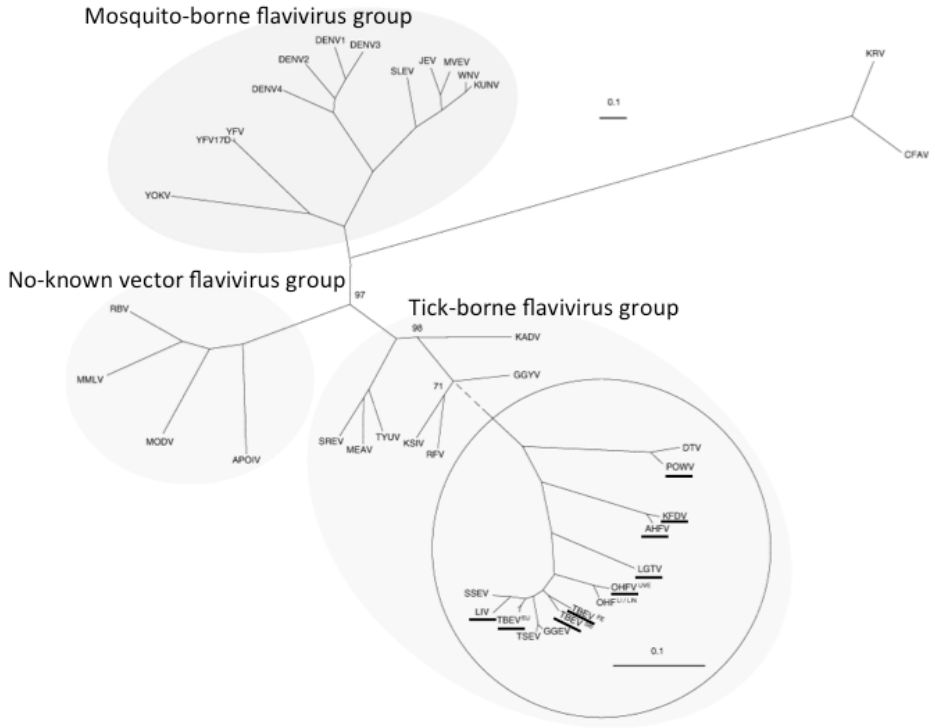

Figure 1. Phylogenetic reconstruction of flavivirus subgroups (Family Flaviviridae genus Flavivirus (53 species): Mosquito-borne flavivirus group, no-known vector flavivirus group and the tick-borne flavivirus group. Of the Mosquito-borne group the *Culex* group is monophyletic, while the group of viruses spread by *Aedes* mosquitos are more divergent. Known human pathogens of the tick-borne flavivirus group are underlined and abbreviated as following: Tick-borne encephalitis virus, TBEV; Louping ill virus, LIV; Powassan virus, POWV; Omsk haemorrhagic fever virus, OHFV; Kyasanur forest disease virus, KFDV; Langat virus, LGTV (no known natural human infections); Alkhurma hemorrhagic fever virus, AHFV (recommended to be included in this group, but is also considered to be a subtype of KFDV).

Figure has been modified from [20]according to [18, 19, 24, 25, 32, 34].

Tick-borne encephalitis virus

Structure, genome and the coding strategy

The flavivirus virions, including TBEV, are approximately 50 nm in diameter and have a smooth surfaced icosahedral shape. The host derived lipid envelope is covered with 180 copies of the two surface proteins, envelope protein (E) and the membrane protein (M), which is derived from precursor protein (prM) by furin cleavage. Inside the envelope is the capsid, which is formed of structural protein i.e. the capsid protein (C) [22, 35, 36].

An approximately 11 kb long single-stranded positive oriented RNA genome acts directly as an infectious messenger RNA. The genome encodes a 4314 amino acids long polyprotein (open reading frame, ORF), which is cleaved by viral proteins post- and co-translationally in addition to the three (E, M, C) structural proteins to seven non-structural proteins (NS) NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [1, 35]. The non-structural proteins have functions necessary for replication, polyprotein processing and and virion formation [35].

E protein has a major role in receptor binding and membrane fusion thus is important for determining the cell tropism and the antigenic properties of flaviviruses [37-39]. In the mature virion the protein forms a dimer, which has three domains. The neutralizing antibodies are induced against several antigenic structures on these domains [40], although NS1 complement fixing antibodies mediating cytotoxic responses by complement activation and anti prM antibodies have also been detected in flaviviruses [41-43]. Besides E protein, 3'-non-coding region, the capsid protein, as well as the non-structural genes NS2B and NS3 have been reported to influence the virulence of a virus strain [44-47].

Phylogeny and distribution

To date there are three subtypes of tick-borne encephalitis virus identified; European (TBEV-Eur, previously known as Central European early summer encephalitis (CEE / FSME) and Kumlinge disease), Siberian (TBEV-Sib, previously known as West-Siberian TBE) and Far Eastern (TBEV-FE, previously known as Russian early summer encephalitis (RSSE) and in China Forest encephalitis - also at present) [28, 41, 48, 49] (Fig. 1-3). Two further genotypes, so far found only in Eastern Siberia, were characterized recently by Tkachev and colleagues [50, 51]. Korenberg and colleagues consider TBEV as a polytypic species and recommend a broader typing system, rather than division of the strains into three subtypes based on their genetics [52]. However the classification of Korenberg and Tkachev is based on probe hybridization, and therefore the typing may not be comparable with the classification based on full or partial sequencing.

The range of TBEV covers largely the geographical range of the main host ticks *I. ricinus* and *I. persulcatus* excluding the southernmost regions. The distribution area of *I. ricinus* reaches from northern parts of Africa through the continent of Europe and the British Isles. *I. ricinus* has not been found in northernmost Fennoscandia

[1, 53, 54]. The distribution area of *I. persulcatus* continues from the North-Eastern Europe to Asia, China and to altitudes further north when compared to *I. ricinus* [52, 55-58] (Fig.2). Within the range of the host ticks, TBEV is found focally where climatic, geographic, vegetation and host population dynamics are suitable for the maintenance of the virus [59-61]. Each focus serves as an autonomous parasitic system characterized by the local abiotic and biotic factors [52].

Figure 2. Map of TBEV distribution modified from [1] according to [4, 5, 62-66].

Tick-borne encephalitis virus spends most of its "life-time" in ticks. The sparse generation cycles of ticks, and the low virus replication frequency of TBEV in ticks determines its evolution and ecology. Hence, in contrast to high mutation frequency generally occurring in RNA viruses and mosquito borne flaviviruses, tick-borne flaviviruses evolve slowly [67] and the strains are stable in each focus [68-71]. In addition, demands on replication in both vertebrate and invertebrate host and the relatively small genome size with little space for variation set strict demands on evolutionary possibilities [72-74].

Australia has been suggested as the origin of *Ixodes* ticks [75] while tick-borne flaviviruses circulating today probably emerged in Africa and evolved during the last 2000 years [20]. According to an established view, TBEV viruses spread from Far-East to Europe, making TBEV-sib a younger type than TBEV-FE and TBEV-Eur with louping ill viruses being the current extreme of the evolvement [19, 67, 68, 72]. However, it has also been suggested, that *Ixodes ricinus*–derived strains have diverged from its ancestor even earlier, than have strains hosted by *Ixodes persulcatus* [20, 70].

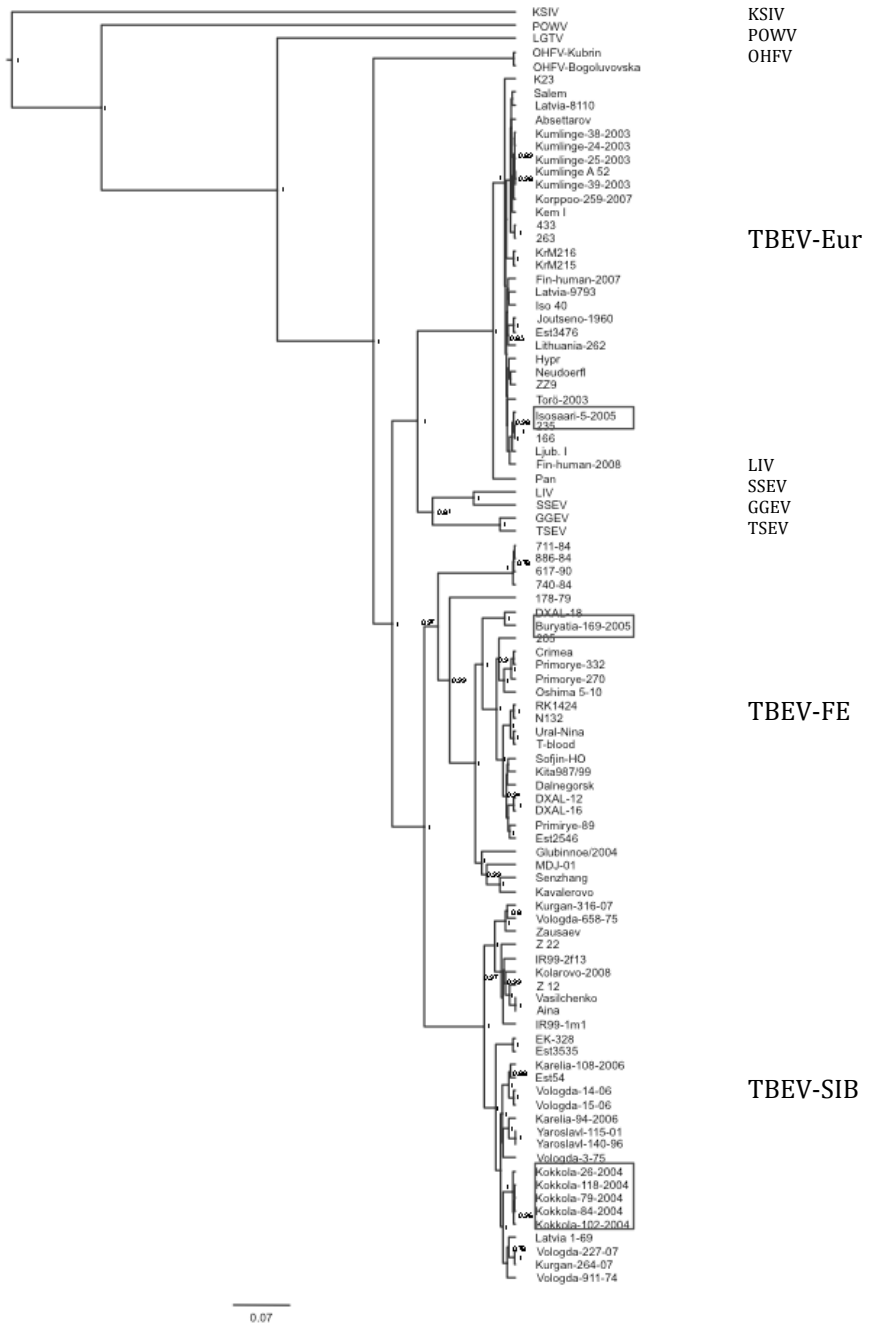

Figure 3. Phylogenetic tree using partial TBEV E gene sequences. Figure is modified from [53] on courtesy of Anu Jääskeläinen and Tarja Sironen. Strains isolated in Kokkola, Isosaari and Buryatia are framed.

The TBEV-Sib and TBEV-FE strains, for which the *I. persulcatus* tick is a main host, are monophyletic (Fig. 3). The genetic diversity of these two TBEV subtypes (in E and NS3 2-9% within TBEV-Sib and 3-13% within TBEV-FE strains) is much higher than among TBEV-Eur (0-4%), mainly hosted by *I. ricinus* [53, 76]. In other studies analysing only the E-gene, the variation has been more moderate [28, 77]. TBEV-FE and TBEV-Sib subtypes have probably been evolving for thousands of years while no geographical clustering is seen for the European subtype reflecting limited genetic variation [70, 71, 78, 79]. The variation between the subtypes was 3,6-5.6%, which is in line with the variation observed within other flavivirus species [28].

Golovljova and colleagues proposed two lineages of the TBEV-Sib subtype: a Siberian and a Baltic, which can be distinguished by unique amino acid substitutions and possibly their pathogenicity in humans (Fig. 3). The geographical boundary of these sub lineages is likely to be the Ural mountains [53, 80] (Fig. 2).

Louping ill is genetically closely related to TBEV-Eur (Fig.2 and 3) and is exclusively transmitted by *I. ricinus*, but causes disease mainly in ungulates and red grouse [28, 66, 81, 82]. Based on complete coding sequence analysis Grard and colleagues [20] suggested that louping ill and Turkish sheep encephalitis virus (now a subtype of LIV) should be combined with tick-borne encephalitis virus subtypes as one species. Recently recombinations between TBEV-Eur and louping ill virus were described, although only *in vitro* or *in silico* [70, 83].

Transmission cycle and maintenance of TBEV in nature

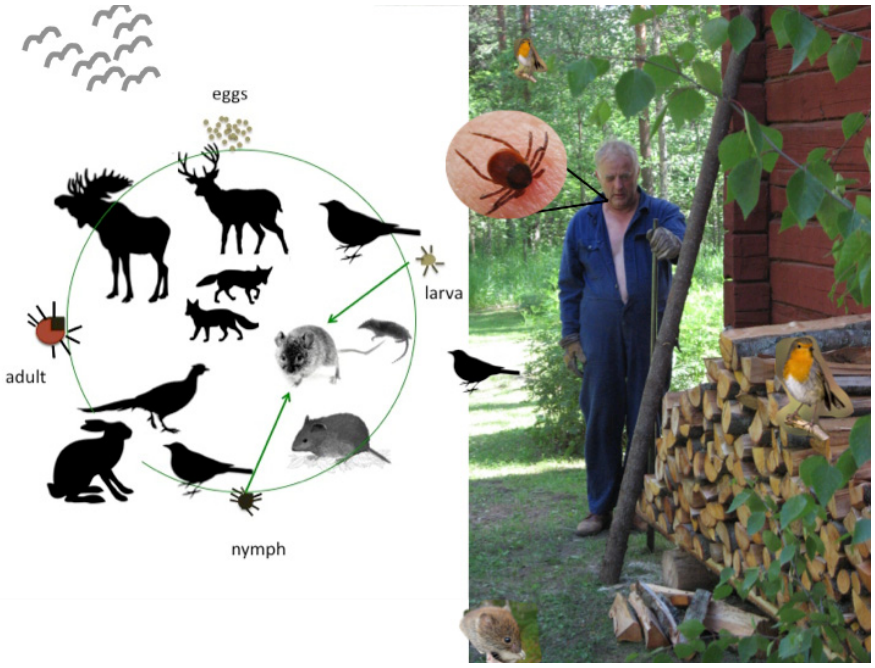

Figure 4. Transmission cycle for TBEV and an accidental host, a holidaymaker at a summerhouse.

Vectors and hosts

The ecology of tick-borne encephalitis virus is largely determined by the ecology of the hosting ticks (Fig 4 and 5) [37]. Ticks act as vectors and constitute the main reservoir for zoonotic TBEV [26, 84, 85].

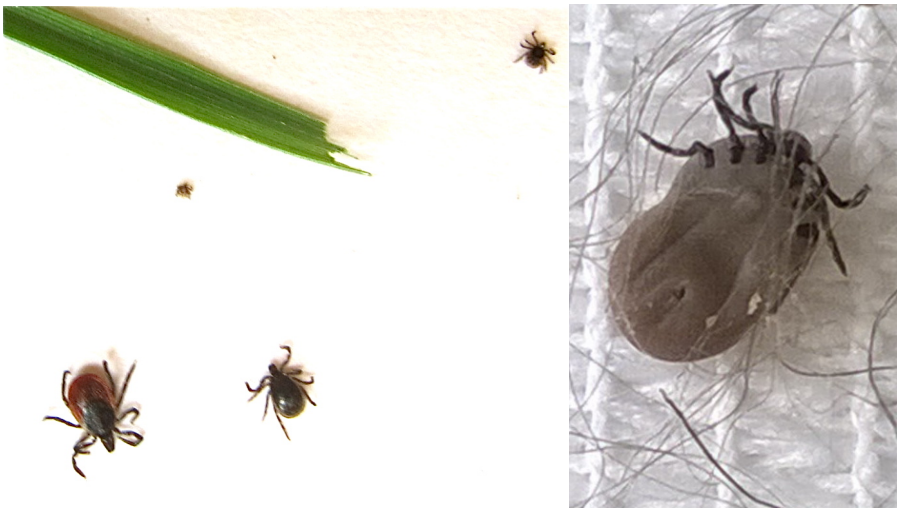

Figure 5 Ixodes ticks. *I.ricinus* female, larva, male and nymph, photo by Elina Tonteri. Engorged *Ixodes* tick, photo courtesy of Pirkko Träskbacka

At least 18 tick species are known to be susceptible for TBEV replication and can efficiently transmit the virus to a vertebrate host [26]. The tick's blood-meal digestion occurs intracellularly in midgut cells and likely determines the vector potential of the species for certain viruses [86]. Local TBEV foci associated with other tick species, than *I. ricinus* and *I. persulcatus* are known [56, 62, 87, 88]. Several tick species are sympatric with TBEV and they could in theory support maintenance of the virus. Nevertheless, *I. ricinus* and *I. persulcatus* are considered as the primary maintenance species due to the favourable seasonality of immature life stages of the ticks and transmission-competent host vertebrates [26, 52, 84, 89].

There are several factors that make *Ixodes* ticks excellent vectors and hosts for tick-borne viruses: long-lived ticks constitute a steady reservoir and stable environment for replication - once infected ticks remain infected throughout their life [1, 26, 37, 86]. The digestive system of ticks is favourable for the viruses: saliva does not digest the blood meal, but the blood cells are ingested as whole and remain unchanged in the tick midgut lumen for long periods to serve as a nutrient reserve for the tick. In addition there are no proteolytic enzymes, which is leaving the viruses time to adapt to tick host and initiate replication in the mid gut wall [26]. Besides the advantages of the tick feeding system in adaptation to tick host, the viruses use tick saliva as immunomodulating medium when infecting vertebrate hosts [90, 91]. On the other hand, the slow generation cycle make the contacts with new hosts infrequent; the

trans-stadial period of the tick is inefficient for the virus [92, 93]. The virus also needs to infect at least one tick cell type that does not undergo lysis in moulting, thus adding selective pressure. The salivary glands are infected depending on the virus before or during the tick feeding on a vertebrate host. In case of TBEV the infection takes place prior to feeding [86].

Ixodes ricinus and *persulcatus* ticks differ in their biotopic preferences [94]. The generation cycles reflect the habitat structure, photoperiod and climatic conditions the tick species need to contend with [86, 92]. However, even geographical strains within the tick species may differ significantly in their life cycles [92]. *Ixodes* ticks acquire only one blood meal per life stage [93] and consequently in optimal conditions they feed once every feeding season, after which they can moult when conditions are suitable. If an individual fails to feed or after feeding moult, it may overwinter and gain the next life stage next feeding season expanding the lifetime. In Sweden, the typical life span of *I. ricinus* varies from 4 years in the south to 6-7 years in northern parts of the country [95]. All life stages of *I. ricinus* can overwinter, but adult females and eggs of *I. persulcatus* populations inhabiting the areas with hardest climatic conditions do not survive the winter and the life cycle of *I. persulcatus* may be prolonged up to six years [92, 96]. On the other hand some *I. persulcatus* populations in the Far East may develop from eggs to nymphs within a feeding season [94] and the *I. ricinus* life cycle may be only two years in favourable conditions [26].

I. ricinus and *I. persulcatus* are considered as generalist ectoparasites with a wide host range of mammals and birds to lizards, although local host specialization does occur. A broad host range also makes them suitable vectors for disease transmission to humans, compared to several other *Ixodes* species feeding only on certain host species [89, 97, 98]. Even if all life stages can feed on different hosts, the larvae feed mainly on small mammals and birds, while nymphs prefer birds and medium-sized mammals. Adult stage ticks attach to medium- to large-sized mammals like ungulates, cats, dogs, fox and hare. Ungulates are important for reproductive female ticks in particular [76, 94, 95, 98-100]. The abundance of adult *I. persulcatus* is fairly continuous from year to year, which shows that in the years of depression of primary host, they switch host successfully to others [76]. A high abundance of large ungulates, most importantly roe deer, which are the most abundant large host in deciduous woods, contributes to the *I. ricinus* population. In the absence of deer, the young life stage ticks of the *Ixodes* move to feed on rodents [95, 101-105].

To serve as a competent host for a TBEV cycle, a vertebrate has to manifest a level of viremia for a tick to contact infective virus particles while feeding. Furthermore the length of the viremia period should be sufficient [84]. Alternatively, vertebrate species can be considered as competent host, if immunocompetent cells are susceptible for TBEV thus supporting non-viremic transmission of TBEV (reviewed below).

Hosts for *Ixodes* tick feeding support the maintenance of TBEV indirectly by ensuring the maintenance of the host ticks [104-107]. However, clinical disease or high or long lasting viremia have not been described in wild large or middle sized mammals, although anti-TBEV-antibodies may be detectable [32, 104, 107]. Mature domestic ungulates do not typically manifest disease nor develop sufficient viremia

in TBEV infection either, but virus is shed to the milk [2] and thereby exposes humans to alimentary infection.

Non-viremic transmission and co-feeding

It was long thought that the transmission of TBEV occurs only through viremic mammalian hosts: the small mammals infected with TBEV by tick bite subsequently get viremic and transmit the infective virus to other ticks [108, 109]. Later it was questioned, whether tick-borne flaviviruses could be maintained in nature only by viremic transmission, as rodent species considered as key hosts for TBEV do not always develop viremia when infected by a tick bite [110] or the viremia is too short and/or not sufficient enough to infect ticks [94, 111]. Furthermore, neutralizing immunity gained in previous TBEV exposure would make these individuals a dead-end host, thus decreasing the number of competent hosts in the population [110].

Transmission of the virus between co-feeding ticks (Fig.6) was first described by Jones et al. with *Rhipicephalus appendiculatus* ticks, that transmitted Thogotovirus from one to another while feeding on a guinea pig [112]. The transmission route was later confirmed also for TBEV for both main tick host species and western and eastern subtypes even in the absence of host viremia (non-viremic transmission/distant transmission) [113-115]. Non-viremic transmission has been also documented for several other tick-borne viruses indicating that it is rather a general mechanism for tick-borne viruses maintenance than an exception [116]. The feeding process of a tick causes an inflammation, which attracts monocytes and neutrophils. TBEV enters the skin from the tick saliva directly when feeding begins. It infects the common immunocompetent cells together with epidermal dendritic cells, the Langerhans cells. The infected cells are then recruited to the feeding site of another tick, bringing the virus to a naïve tick independently of host viremia [117]. Non-viremic transmission is enhanced by the immunomodulation capacity of tick saliva [38].

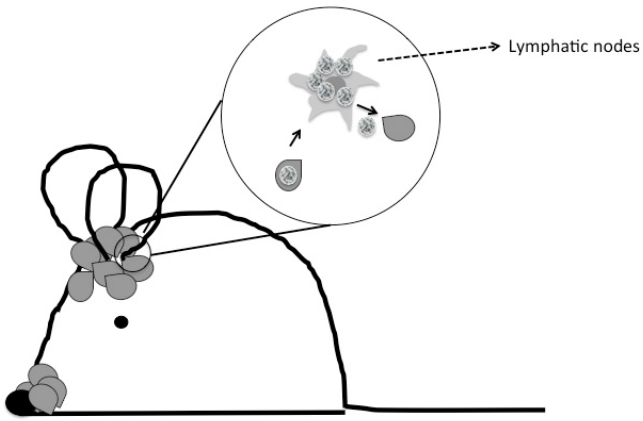

Figure 6. Ticks co-feeding around ears and snout of a rodent leading to non-viremic transmission and possible infection of the host rodent

Competent hosts for non-viremic transmission of TBEV were further characterized by Labuda and colleagues [110, 118]. Potential hosts, *Phasianus colchius* (pheasant), *Erinaeus europaeus* (hedgehog) and *Turdus merula* (blackbird) did not support non-viremic transmission, while *Apodemus agrarius* and *Apodemus flavicollis* mice (field mouse and yellow-necked mouse respectively) developed only a low level or no viremia, but ticks were infected efficiently [118]. *Myodes glareolus* voles (bank vole) both fed ticks poorly and non-viremic transmission occurred less frequently. TBEV (Eur) was shown to transmit between co-feeding of *I. ricinus* ticks even on immune *A. flavicollis* and less efficiently on *M. glareolus* [110, 118]. *Microtus pinetorum* voles (pine vole), whose range do not overlap with TBEV-endemic areas, developed a high viremia, but they died before the tick engorgement was completed [109, 114].

For non-viremic transmission to occur, the ticks need to aggregate coincidently on a transmission competent host to co-feed. For TBEV maintenance, co-feeding of larvae and nymphs is crucial. The activity of the young life stage ticks is parallel when temperature is rising rapidly in spring, as for rapid cooling in fall predicts parallel activity in following spring [104, 119]. Humidity close to the ground largely determines the questing activity of larvae. On the other hand, low humidity in the upper questing height of nymphs must bring the nymphs closer to the ground to feed on the same hosts than larvae [120, 121]. Larvae tend to aggregate, so several larvae are likely to attack an individual host mammal [122].

Even if the host range of *Ixodes* ticks is wide, locally the feeding distribution of ticks on their hosts is typically over-dispersed [122]. A few mammalian species and within their populations approximately 20% of the individuals carry the 80% majority of the ticks infested. These figures are well supported by findings in wild rodents [84, 123]. Furthermore, ticks aggregate to feed on certain parts of the host body (Fig.6) [122, 124].

In Central European woodlands the two most abundant rodent species, and which are competent for non-viremic transmission of TBEV, *A. flavicollis* and *M. glareolus* are heavily infested by immature stages of *I. ricinus* [59, 125-128]. However, the host preferences differ according to the availability [122] and other *Apodemus* mice and *Microtus* voles are also infested by immature *Ixodes* ticks [129]. *M. glareolus* dominate in the *I. persulcatus* foci in pre-Ural regions (Fig. 2) [130]. In the eastern range of the TBEV endemic area insectivores are most abundant small mammals, but *Myodes rutilus* (northern red-backed vole) and *Apodemus agrarius* harbour most immature *I. persulcatus*: *M. rutilus* harbour more larvae, while more nymphs are found in on *Apodemus* mice [131, 132]. Furthermore, distribution of ticks on *Sorex araneus* (Eurasian shrew or common shrew) is more scattered and thereby less favourable for non-viremic transmission than that on the competent rodent hosts [122].

Mature individuals are more infested by *Ixodes*-ticks than juveniles and higher body mass correlates positively with tick burden [100, 123, 129, 132]. Old males have been shown to be most infested at least for *M.glareolus* and *M. rutilus* [100, 132]. Testosterone impairs the innate and acquired resistance of voles to parasitizing ticks, thereby increasing the number of parasitizing and engorging ticks. Sexually active males also have a wider activity range than immature voles or females and therefore bigger chance to meet a micro-geographical spot with high density of questing ticks [100, 133, 134]. Host odour may attract ticks [93] and it has been

suggested, that host specialization by questing height and activity may develop in *I. ricinus* populations [98].

Rodent host immunity to ticks

Besides mechanical grooming, hosts may protect themselves from blood sucking ectoparasites by acquiring immunity to tick saliva antigens [135]. The resistance affects the success in attachment, duration of feeding in case of successful engorgement, and therefore size of the tick and production of ova. Those ticks, which fail to engorge fully may not moult, but die [125, 136].

M. glareolus has been shown to acquire resistance to *I. ricinus* larvae while *A. falvicollis* is rather immunosuppressed by an *I. ricinus* bite. In addition *M. glareolus* voles develop an inflammation at the site of tick bite [125]. *Apodemus*-mice are indeed more likely to be infested by *I. ricinus* than *Myodes* voles even if behavioural risk to tick attachment is excluded [100, 122, 123, 129, 132]. However, in a long-term study in the Novosibirsk area in Russia *M. rutilus* voles were significantly more often infested with larvae and nymphs of *I. persulcatus*, than field mice. *Sorex araneus* only harboured larvae [131, 137].

Transovarial transmission

TBEV transmits transovarially in *Ixodes* ticks [138, 139]. Transmission frequency is low, fewer than 5% of hatching larvae are infected, making this transmission route supportive rather than essential for TBEV persistence in nature [52, 139]. Low transmission in a supportive maintenance system is, nevertheless, favourable for the virus as a high rate of transovarial transmission might cause deleterious mutations. 350-5000 of eggs may be laid by *I. ricinus* or *I. persulcatus* females in a certain spot. Even if only a few hatching larvae are infected, all are attaching to the same host rodents and thus the few infected ones may infect several others when co-feeding [77, 96, 138]. The studies of frequency and thus importance of transovarial transmission in *I. ricinus* compared to *I. persulcatus* are controversial [139-141].

Vertical transmission and persistent infections in small mammals

Vertical transmission of TBVE-Sib between *M. rutilus* generations has been shown in the wild and experimental settings. In the latter, sexual transmission has also been reported [142, 143]. TBEV may also persist in host rodents, but the mechanism and the importance of persistence for maintenance of the virus is unclear [126, 131, 144]. Hibernation and its affect on TBEV persistence is a forgotten branch in TBEV maintenance studies. Even if hedgehogs do not support the non-viremic transmission of TBEV [118], the virus persists in hibernating hedgehogs, which in spring subsequently develop viremia [141, 145].

Host abundance, biodiversity and dilution hypothesis

In temperate deciduous forests rodent populations are stable (non-cyclic), but outbreaks are driven by heavy seed crops (masting) [128, 146]. *Apodemus* mice are most abundant in high growth forests with suitable horizontal layers and heaviest

masting, while *M. glareolus* is more a generalists species and is found more widely throughout the deciduous and coniferous region [105, 128, 147, 148]. In a long-term study in Poland, masting affected breeding and numbers of *A. flavicollis* more, than what was seen in *M. glareolus*, which species is more dependent on the herb layer. Masting was followed by winter breeding in *Apodemus*. On the other hand in Denmark, the opposite was seen namely the winter breeding of *M. glareolus* in mast years. This was explained by self-regulation of breeding in the mouse population [146]. However, winter mortality is driven by other factors than food resources - weather or social behaviour and importantly by predation. In winter, predators target to rodents in the lack of other prey such as birds [128]. While rodent peaks are followed by heavy masting, the predator species balance affects the decline of rodents.

In deciduous forests of the taiga region, north of 60°N, microtine rodent populations are cyclic. The cycles are driven by predation, although food resources and diseases may be important factors [149, 150]. The cycle length (3-5 years), the amplitude or the fluctuation and interspecies synchrony increases northwards, which has been explained by specialization of the northern predators (for example small mustelids and several owl species), in contrast to dominance of generalist predators (for example common buzzard, fox, bobcat, domestic cat, marten, tawly owl, corvids) and generalized predation in temperate and the southern boreal region [150, 151].

Furthermore, the amplitude of the changes in population density is lower in Southern Scandinavia (temperate zone), than what is seen in the cyclic variation in Northern Scandinavia (boreal zone), however the main difference may not be the peak densities after masting, but the lack of the very low-density periods in the southern area [146].

The dilution effect refers to the situation, where burden of a zoonotic pathogen is eased on certain species or populations due to changes in population density or biodiversity – more wildlife may lead to less mosquito or tick bites in humans and thereby to fewer infections of pathogens spread by these arthropods [13]. The dilution effect may also affect the maintenance of a pathogen in a vector-host population system. For pathogens such as Puumala virus spreading from rodent to rodent, the dilution effect refers to the decrease in virus prevalence due to decrease in intraspecies contacts of *M. glareolus,* reflecting a change in rodent species abundance [152]. The availability of competent hosts for non-viremic transmission is considered to be the key factor for TBEV maintenance. However, dense host rodent population may reduce the possibility of tick-to-tick transmission as tick bites are distributed to more individuals [127]. Also, high density of low-competent hosts or incompetent dead-end hosts, such as deer, has a negative effect on the number of co-feeding ticks on competent hosts. On the other hand, a long-term absence of sources for a tick blood meal may lead to depression of the tick population and TBEV prevalence [103, 104, 153].

Other factors

Borrelia- and TBEV-infections may alter the tick questing behaviour [94] and infection of the tick with borrelia bacteria may also direct the host preferences of the tick [98].

Pathogenesis and infection kinetics of TBEV infection

Tick-borne encephalitis virus is neurotropic [144, 154, 155]. However, the infection initiates extraneuronally as a localized skin infection at the tick feeding site and the first targets for TBEV are the immunocompetent cells (Fig.6). Insertion of the tick hypostome into the epidermis is followed by infiltration of neutrophils and monocytes. However, tick saliva includes several immunomodulating compounds that interfere with the host's immune responses, namely complement activation, phagocytosis, natural killer cell functions, T-lymphocyte functions and cellular messaging thus providing an advantage for the virus transmission [26, 91, 117, 136, 156, 157]. Migrating epidermal Langerhans cells are susceptible to TBEV replication and carried by them, the virus is transported to the lymphatic system via local lymph nodes [117]. TBEV can also multiply in infiltrating macrophages and neutrophils in cell cultures and in mice [156, 158, 159]. Neutrophils have a promoting role for infection in mice [117, 157, 160].

Subsequent viral release to blood manifests in viremia and infection of extra neuronal organs, especially those of reticulo-endothelial system i.e. spleen, liver and bone marrow [3, 161]. The cell receptor target for TBEV E-protein is still unknown. It has been suggested, that the receptor is either some abundant in wide range of animal tissues, such as heparane sulphate [44, 162, 163] or several different molecules can act as receptors for TBEV binding [164]. Extra-neuronal target organs for TBEV replication are poorly characterized.

Neuropathogenesis of TBEV includes the ability of the virus to enter the CNS, which requires effective replication outside of the CNS to avoid neutralization by host immune response, and the ability to replicate in the neurons i.e. neuroinvasiveness and neurovirulence, respectively [37, 46, 47, 164]. Changes in neuropathogenesis are thus the result of various factors [164]. The mechanism of TBEV access to the CNS via blood-brain barrier (BBB) remains unknown. Replication in CNS endothelial cells and subsequent infection of astrocytes, passive diffusion of the virus particles, transcytosis along infected immune cells, entry via peripheral nerves and invasion of the olfactory epithelium have been suggested [3, 155, 165, 166]. Furthermore, overproduction of chemokines and cytokines in late stages of TBEV-infection in CNS alters the permeability of BBB [167-169].

Neurophagia, neuronal necrosis, and direct cytopathic effects are seen in experimental infections in mice and monkeys and in post mortem analysis of human and dog brain tissues [154, 164, 170-175]. However, several disease models suggest that it is rather the host immune system that contributes to disease progression than neuronal apoptosis or necrosis due to virus replication [167, 168, 176]. While successful production of neutralizing antibodies early in infection protects the host from neuroinvasion and fatal outcome in mouse model [177], extensive inflammatory reaction with extensive increase in proinflammatory cytokine levels in neuroinfection may cause immunopathogenesis and death [167, 168, 178]. In animal models the infection route also seems crucial for the outcome. Subcutaneous infection produces mainly mild or non-symptomatic disease, while intracerebral infection causes encephalitis or even death at least in monkeys, sheep and mice [33, 161, 179, 180].

Clinical picture

In humans, wide individual variation in clinical outcomes of TBE is seen according to e.g. age and the host genetic factors [181-183]. The disease course may vary also according to the causative TBEV-subtype. 70-98% of infections remain subclinical. In the individuals, that develop a clinical disease, the most common unspecific symptoms of the viremic stage are general malaise and fatigue, fever and aches. Later the disease may develop to CNS infection manifesting as mild meningitis to severe encephalitis and myelitis. The symptoms are typical for an acute viral meningoencephalitis: headache, impaired consciousness, ataxia, tremor and paralysis, in case of TBEV especially in the upper limbs and neck [1, 41, 184-189].

TBEV-Eur manifests the mildest form of TBE. A biphasic course (Fig. 7) is described to be characteristic of the disease caused by this subtype, although it should not be considered as rule as in 25-50% of cases such course is not observed [189, 190]. One third of the patients with TBEV-Eur infection manifest CNS inflammation (meningitis, encephalitis and/or myelitis). The mortality rate is low, 1-2%, but approximately one third of the patients with neurological damage suffer from one or several sequelae impairing the quality of life: cognitive disorders, headaches, ataxia, tremor and paralysis. The severity of the sequelae correlates with the severity of the acute stage. Tendency to develop severe disease form and sequelae increases with age [184, 188, 189, 191]. Of the three subtypes TBEV-FE causes most often a severe encephalitis and damage in CNS. Sequelae are common, only 25 % of the patients recover completely, and 20-40% of the infections are reported to be lethal [188]. In contrary to TBEV-Eur, TBEV-FE targets the young age groups. The disease course in TBEV-FE-infection is mainly monophasic. TBEV-Sib infections are less severe, mainly meningeal and febrile forms, with either bi- or monophasic course and mortality of 1-8% [1, 41, 186]. Rare chronic, progressive or recurrent forms of clinical TBEV with or without a previous acute phase, and sometimes even lacking antibody response, have been associated with the TBEV-Sib in humans and monkeys, although the findings are considered controversial by many researchers [1, 41, 179, 185, 188]. Also, the high proportion of the persons with severe clinical outcomes in TBEV-FE and partly TBEV-Sib endemic areas may be biased due to the different diagnostic criteria and reporting systems from that of TBEV-Eur endemic area. The claim is supported by the outcome of a prospective study in Lithuania, where complete recovery was reported in less than 25% of the patients with severe encephalitis caused by TBEV-Eur [188].

Diagnosis and prevention of TBE

Anatomical and functional imaging by magnetic resonance imaging (MRI), single-photon-emission computed tomography (SPECT) and electroencephalogram (EEG) reveal abnormalities with part of the TBE infections. At the initial phase, serum thrombo- and leucocytopenia is considered typical for acute TBE and in the second phase two-thirds of the patients manifest moderate pleocytosis in cerebrospinal fluid (CSF) with change of the dominance of polymorphonuclear cells to mononuclear cells exclusively. Also moderate increased albumin level in CSF compared to serum indicate damage in blood-brain barrier and thereby CNS infection. However, the above-mentioned findings have no diagnostic value, but are rather supportive [192, 193]. Specific laboratory diagnostics of TBE relies on demonstration of anti TBEV-

antibodies by serological methods, of which enzyme immunoassay detections of antibodies are most commonly used [194]. Viral RNA can be detected and virus is isolatable only in the early phase of the illness. However, TBE is rarely diagnosed during the viremic phase by mild generalized flu-like symptoms, but later, when CNS symptoms manifest and viremia is cleared [3, 184, 193]. Anti-TBEV immunoglobulins M and G (IgM and IgG) develop from the onset of neurological symptoms onward. IgM antibodies are usually detectable in first serum sample, in case of manifest infection and IgG within 2 weeks. In CSF, specific IgM antibodies are detectable from the first sample or by latest 9 days from the onset of the symptoms. Intrahecal production of IgG can be detected by day 15 after the onset of symptoms [195] . IgM response last approximately up to eight weeks, thus presence of IgM indicates an acute infection (Fig. 7). Netralizing anti-TBEV-IgG antibodies provide protection against reinfection, normally for the rest of the life. The IgM diagnostic tests are sensitive to non-specific false positive results and the finding has to be confirmed with another test detecting also IgG-antibodies [1, 193, 195]. Notably, in rare chronic infections though IgM antibodies may persist for more than a year and an IgG-response is not always seen [43]. In case of vaccination failure, the IgM response is delayed even if IgG is detectable, often in high titers. A second sample to confirm the rise of IgM titer or alternatively detection of specific anti-TBEV-antibodies in CSF is needed in such cases for diagnosis of TBE [193, 196].

The flaviruses are similar in their genomic structure and morphology and therefore in their antigenic properties, causing cross-reactions in serological testing [39]. Previous travel (to JEV, YFV, dengue or WNV endemic areas) and vaccination history (JEV and YFV) of the patient needs to be considered when diagnosing TBE. In case of suspicion of a cross-reaction, the results should be confirmed by a neutralization test [42, 193].

There is no specific antiviral or immunomodulatory treatment available for TBE, and hence the treatment is symptomatic. TBE is preventable by wearing clothing to protect from tick bites, and additionally through immunization [1, 184]. Two vaccines with a similar structure based on closely related strains are available in the European Union: TicoVac® (also with tradename FSME-IMMUN®) and Encepur®, which both include a formaline-inactivated strain of TBEV-Eur. In addition two vaccines, TBE-Moscow® and EnceVir®, are in the market in Russia and neighbouring former Soviet countries. In China there is a vaccine available, which is based on strain Senzhang. The latter three represent the TBEV-FE subtype [56, 197]. According to several studies, all vaccines provide cross protection between all TBEV subtypes [196, 198-200].

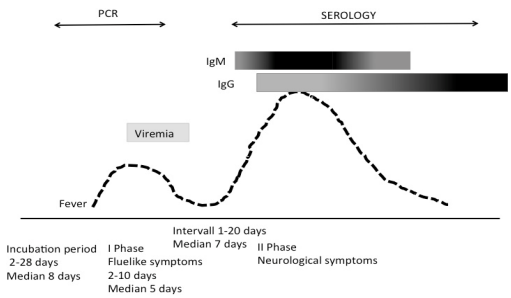

Fig 7. Biphasic TBE; disease course and diagnostics according to [1]

Materials and methods

Wild small mammals (I and II)

Small mammals were trapped in two subsequent winters 2008 and 2009 in two earlier known TBEV-endemic foci: Isosaari, Helsinki [79] and in Kokkola [78]. We also carried out trappings during tick-feeding season in suspected new TBEV-foci: Simo, Varkaus, Kotka, Kuopio and Sipoo (Fig 8, Table 2). Collection sites were chosen by earlier known sites of tick bite indicated by TBE-patients (interviews by the National Institute of Heath and Welfare, NIHW).

Snap traps were set in the evening and collected in the morning. Pieces of rye bread were used as bait (Fig. 8). The animals were moved onto dry ice immediately after collecting and stored in -70°C until dissection. Brain, spleen, liver, lungs and kidneys were collected for further analysis and stored in -80°. Heart extracts were dissolved in 300 µl (rodents) or 200 µl (shrews) of phosphate buffered saline (PBS).

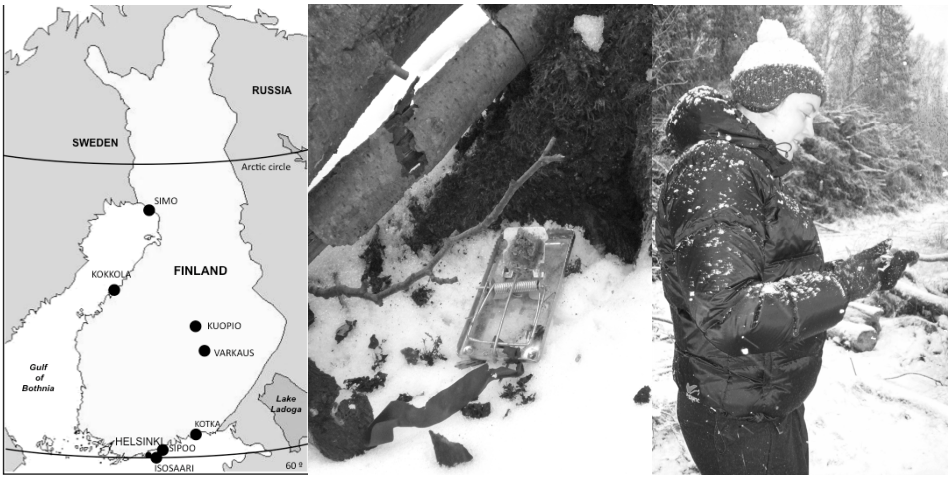

Figure 8. From left to right: Trapping sites, a trap with rye bread bait and a trapper setting a trap in Isosaari 2009. Trap picture courtesy of Anna Enzerink.

Tick collection in Simo (II)

Ticks were collected by flagging in Simo during June 23-26th 2009 (Table 2). Ticks were further handled in 51 pools of 1-3 ticks.

Cell lines (I-III)

Interferon I (α and β) deficient African green monkey kidney epithelial cell line Vero E6 (ATCC: CRL-1586) was grown on minimum essential Eagle medium (MEM) supplemented with 5 to 10% fetal bovine serum (FBS) and antibiotics and with or without HEPES and antimycotic Fungizone. Syrian golden hamster kidney fibroblast

cell line BHK-21 S-13 (ATCC: CCL-10) was grown on minimum essential Eagle medium (MEM) (Gibco/Invitrogen) supplemented with 5% fetal bovine serum (FBS), antibiotics and HEPES (Gibco/Invitrogen).

Viruses (III)

Virus strains used for experimental infection of bank voles were TBEV-Sib, Kokkola-8 [78]; TBEV-Eur, Isosaari-5 and TBEV-FE, Buryatia-169 [53] (Fig. 3). Strains were first isolated from ticks by our group and subsequently passaged once in suckling NMRI mice *in vivo* by intracerebral inoculation. Virus titers were determined by tenfold cell culture titration (on Vero E6 cells) until CPE was seen and using plaque titration assay with agarose overlay medium added after 1 hour infection and crystal violet staining. In addition, we used a rapid fluorescent focus inhibition test (RFFIT) method (on BHK-21 cells) earlier described by Vene and colleagues [201]. Stock dilutions of the three strains were equalized for their fluorescent focus unit content.

In vivo models for TBE infection kinetics and persistence (III)

Inbred bank voles (*Myodes glareolus*) from a colony maintained by Astrid Fagraeus Laboratorium were used as *in vivo* model for acute TBEV infection kinetics (up to 25 days post infection) and persistence of the virus (up to 168 days post infection) (Table 2). Animals were maintained in isolator cages under BSL-3 conditions in groups of 2-3. Food and water was provided *ad libitum*.

The experimental settings (excluding histopathological examination) and infectivity of the strains in bank voles were tested prior to the actual experiment in a pilot experiment up to 22 days post infection. All together 24 animals were infected with different dilutions of the stock dilutions: TBEV-Eur: 2x -7 -6, -4 and -3, 1 x -1; TBEV-Sib: 2x -7 -6, -4 and -3, 1 x -1; TBEV-FE: 2x -7 -6, -4 and -3, 1 x -1. An additional two uninfected negative controls were infected. Blood samples were drawn on day 15.

In the main experiment, blood samples were drawn throughout the experiment and whole blood was taken upon euthanizing the animals. Brain, lung, liver, spleen, kidney and uterus of female animals were collected for molecular and histopathological examination. In addition, urine and excreta of animals after 54 dpi was collected along with other sampling.

Ixodes ricinus from a pathogen free laboratory colony were kindly provided by Natasha Rudenko. 3 to 4 adult female ticks were set on each sedated bank voles upon arrival and 8 days before the experiment termination (160 dpi). Nymphs were left in +4°C and 10 of them were set on each non sedated bank voles prior to sample collection.

At 168 dpi, all bank voles left were euthanized and a piece of skin around the snout, the only spot where any ticks were found attached, was cut of and set on a clean petri dish with a stalk of grass. 2-3 attached an/or fed ticks were found on 4 of 9 animals left representing all 3 virus subtypes used for infection. All attached ticks were

nymphs. Ticks detached in 1-3 hours and they were subsequently homogenized in Dulbecco+0,2% BSA prior to further molecular analysis of TBEV infection.

Human serum screening (IV)

We obtained 1957 clinical serum samples of patients with neurological infections with unknown aetiology that had been sent to the laboratory of Hospital district of Helsinki and Uusimaa (HUSLAB) from around Finland for screening of human herpesvirus 6, herpes simplex, varicella zoster and *Mycoplasma pneumonia* but had been found negative for these pathogens. Patients with previous TBE diagnosis were excluded.

Samples were chosen as comprehensively as possible from each year to represent the feeding season of ticks and previously known period of patient cases in Finland as following: 1997, 1. August to 31. October; 2005 and 2006 15. May to 31. August; 2007, 20. June to 23. August; 2011 and 2012 1. June to 31. October.

TBE-cases in Finland 1995 / 2007-2013 (I, II and IV)

TBE is a notifiable disease in Finland. Positive serological results are, since 1995, reported to the Finnish National Infectious Disease Register by the two diagnostics laboratories (Turku and Helsinki). Cases are registered according to the hospital district of the treating hospital. To ensure that cases meet the criteria for TBE case definition by ECDC and to further gain information including putative geographical place of infection, the date of first symptoms and vaccination history, National Institute for Health and Welfare (NIHW) collects the medical records and if possible contacts the patients. Above-mentioned data of NIHW, available since 2007, was used in present study to locate the geographical places of the TBE infections and to survey TBE epidemiology in Finland.

Virus isolations (I-III)

Samples for virus isolation experiments were chosen from those positive in screening for TBEV-RNA using real-time-RT-PCR and /or nested RT-PCR.

Virus isolations were carried out in 0-3 days old suckling NMRI mice. 20 µl of rodent organs or ticks suspended in 800 µl Dulbecco's PBS including 0,2 % BSA (Table 3) were injected intracerebrally to each puppy in a litter. Infected animals were followed up to 14 days post infection or until any symptoms were observed. Mice were anaesthetized by isoflurane inhalation prior to putting down (according to ethical statement). The brains were stored in -70°C until further analysis.

We also performed sequential blind passage of Vero E6-cells up to 9 passages (III) described by Achazi and colleagues [144]. For samples of 2 wild winter trapped rodents (Kokkola and Isosaari 2008) we made an isolation attempt on Vero E6 with only 2 passages (Table 3).

Molecular analysis (I-III)

The samples of ticks, mammal organs and excreta were homogenized using Magnalyzer (Roche Applied Science) (I-III) or a Tissuelyser (Qiagen) (III) device. RNA and DNA were extracted using Tripure reagent (Roche) according to the instructions of the manufacturer except for serum samples and excreta for which QIAamp Viral RNA Mini Kit (Qiagen) was used. The later method was also used to extract RNA from cell supernatants when confirming the cell culture infections.

Tick, mammal and cell culture supernatant samples (I-III) were screened by real-time RT-PCR described by Schwaiger and Cassinotti [202] with following modifications: 150 nmol/l of forward primer (instead of 50 150 nmol/l), 500 nmol/l reverse primer (instead of 300 nmol/l) and 400 nmol/l of probe (instead of 200 nmol/l).

In addition nested NS5-RT-PCR [203] modified by Anu Jääskeläinen and colleagues [78], 5'NCR-RT-nested PCR [204], partial E RT-nested PCR [53, 205] and pan-flavi-NS3-PCR [20, 206] were used to confirm the positive results of real-time RT-PCR for the original samples and to gain nucleotide sequences from original samples or mouse brain isolates (I and II).

Prior to sequencing, samples were purified using ExoSAP-IT (Amersham Biosciences) and/or QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions. In study I also pGEM®-T Vector Systems (Promega) following with QIAprep Spin Miniprep purification (Qiagen) was used.

Sequencing reactions were run on ABI3130xl sequencer using Big Dye Termination kit by Applied Biosystems according to manufacturer's instructions. The same primers as in PCR were used except for partial E-gene, of which sequencing was also done from the middle using forward primer 5'CGCAAAACTGGAATAACGC and reverse primer 5'-CATCTTGACAGCGTGAGGAG.

Morphological determination of the tick species was confirmed by *Ixodes* mtDNA PCR [207].

Phylogenetic analysis (I and II)

A 165-nt stretch of the NS5 gene obtained from the original winter trapped bank vole sample (GenBank accession no. GU458800) was aligned manually with published TBEV sequences available in GenBank to determine the phylogenetic position in terms of the three subtypes described by Ecker and colleagues [28].

To determine the phylogenetic clustering of the isolated virus strains from Simo (II) Partial E (1298 nt) and NS3 (604 nt) gene sequences (GenBank accession HQ228014-HQ228024) were analysed as described by Jääskeläinen et al. [208].

Serology (I-IV)

Wild mammal samples collected in Kokkola and Isosaari in 2008 (I) were first unsuccessfully analysed using hemagglutination inhibition test [201]. The latter and

samples from year 2009 (I) in addition to samples collected in Simo (II) were then analysed using immunofluorescence assay (IFA). Vero E6 cells infected with Kumlinge A52 strain [209] mixed equally with uninfected Vero E6-cells were used as antigen and conjugated with polyclonal rabbit anti-mouse fluorescein isothiocyanate conjugate (Dako, Glostrup). Samples were viewed with Olympus BX51 fluorescence microscope with 20× or 40× objective and 10× oculars. We also studied samples from sequential blind passage on Vero E6 cells by IFA according to the description above.

To follow the TBEV infection kinetics and anti-TBEV antibody response in bank voles commercial IMMUNOZYMH FSME (TBE) IgG All Species kit (Progen Biotechnik GmbH) was used according to the manufacturer's instructions (III).

To screen anti-TBEV IgM-antibodies in patient sera, μ-capture IgM-enzyme immunoassay (EIA) earlier described by Jääskeläinen et al. [194] was used with a modification. Instead of peroxidase conjugated anti-TBEV-Mab, we used anti FSME monoclonal antibody 1786 [210] supplied by Matthias Niedrig and colleagues, Robert Koch Institute with subsequent peroxidase conjugated donkey Anti-Mouse IgG antibody (Jackson Immunoresearch, West Grove, PA, US). The Sf9-bac-TBEV-PrME antigen was produced in house by us. Samples found positive in μ -capture IgG-EIA were studied for total anti-TBEV antibodies with in-house hemagglutination inhibition test [201]. As an exception, samples from 1997 were screened using IgM with EIA (Progen Biotechnik GmbH, Heidelberg, Germany) and with an in-house HI-test [201].

Other methods (III)

Histopathological and immunohistological analyses were done as described in [211]. Statistical analysis was conducted as described in [211].

Ethics statements (I-IV)

All animal handling was done following the guidelines of The Swedish Institute for Communicable Disease Control (at present The Public Health Agency of Sweden), Solna, Sweden and the University of Helsinki, Helsinki, Finland.

The experimental studies on bank voles (III) were approved by the authority for animal study ethics in Stockholm (#N419/10 and 339/07). Ethical permit for virus isolation in mice (I, II and III) was approved by authority for animal study ethics under the County Administrative office of Southern Finland (decision number STH502A, ESLH-2008-06558/ym-23). According to the Finnish Animal Experiment Board trapping animals with snap traps that instantly kill the animal is not considered as an animal experiment and thus no permit is needed (I and II), referred in [152]. All trapping was carried out with permission of land owners.

Ethical approval (HUS §32/2013) for human sample screening (IV) was concened by Huslab responsible authority. The data for epidemiological nominator was analysed with approval of ethical board of the National Institute of Health and Welfare: THL/402/5.05.00/2014.

Aims of the study:

-To study the ecological factors important for the establishment and maintenance of TBEV foci in Finland in the boreal region and to compare maintenance of TBEV in previously confirmed TBEV-Eur and TBEV-Sib foci

-To study TBEV infection kinetics in the natural host in acute and prolonged settings using all three subtypes of the virus

-To survey the development of incidence and clinical alertness for TBE and to determine geographical distribution of the infection sites of TBE in Finland

Results and discussion

Rodent hosts for TBEV in Finland

The studies of non-viremic transmission in *Apodemus* mice and *Myodes glareolus* voles have contributed mostly to the understanding of competent host rodents for TBEV. In Central Europe, *Apodemus* mice are considered to be the most important hosts for TBEV maintenance [127]. They have similar habitat preferences and are most infested by *Ixodes ricinus*. Furthermore, *Apodemus* mice are considered to support non-viremic transmission and co-feeding most efficiently, whereas *M. glareolus* gain resistance to ticks, impairing the feeding [123, 125]. In Novosibirsk, Western Siberia, *M. rutilus* is the most abundant small mammalian species in TBEV foci and TBEV-antigen is relatively more prevalent in *M. rutilus* and *S. araneus* than in *Apodemus agrarius* [137]. Thus maintenance factors may differ between the biomes.

We trapped mammals in seven sites, where human infections had previously been known to occur (Fig. 8, Table 1): Simo, Kokkola, Isosaari, Kuopio, Kotka, Sipoo and Varkaus. Our observations suggest that in these TBEV foci lying in southern and mid boreal regions [212], the main rodent species responsible for ensuring the TBEV circulation are *Myodes glareolus* and *Microtus agrestis* (field vole). These are the dominant small mammal species in the TBEV endemic foci studied and TBEV infection was continually shown in these species. In addition, *S. araneus* and *Sorex caecutiens* (Laxmann's shrew) were trapped relatively frequently, but only one individual was found positive for TBEV in any tests. However, our trapping method of having rye bread as bait may attract more rodents than insectivores thus causing bias to the observed species balance.

Myodes glareolus is the most common rodent species in Fennoscandia. The species is found in the whole of Finland except for the outer archipelago and northernmost Lapland. Other highly abundant species, *Microtus agrestis* prefers hay fields, meadows and logging areas and is found all over Finland [147, 213]. *M. glareolus* voles in turn are found in forests of all ages and especially in the absence of *Microtus agrestis* also in clear cut areas and peatlands, but are most abundant in old growth forests rich with food sources and shelter [147, 152, 214, 215].

Apodemus mice habit hemi- and southern boreal regions in Finland [212, 213, 215]. Even if *M. glareolus* and *M. agrestis* dominated in TBEV foci studied by us, it cannot be excluded, that *Apodemus* mice support TBEV maintenance in some foci especially in South-western Finland. Indeed, humid grove habitats preferred by mice are also in favour of ticks. Shrews are common in the whole country, but little is known about their tick infestation in Finland or ability to support non-viremic transmission of TBEV.

M. glareolus voles have been shown to be able to gain resistance to ticks thus hindering the TBEV transmission between the ticks. However, the host rodent population resistance to ticks may vary in different areas. Tälleklint and Jaenson [100] suggest positive selection for host immunity to ticks in areas, where *I. ricinus*

has been co-habiting for long, whereas in recent foci it has not developed yet. The ability to develop resistance to ticks is heritable, at least in cattle [125]. In Sweden acquired resistance was found relatively unimportant [100, 125]. In areas, where *I. ricinus* has been presented relatively recently, strong selection for acquired resistance may not have been developed. Also, in the northern regions, where tick-feeding season is short, the pressure for inherited resistance is low. The seasonal pattern of rodent breeding differing between the temperate regions and northern taiga region may be crucial – in spring there are few individuals with acquired resistance left [100, 216]. For individuals, the number of ticks feeding on the host rodent may contribute to the level of resistance [94, 132].

Rodent resistance to ticks affects TBEV maintenance in terms of tick population survival, as partially fed ticks may not be able to moult. During the blood meal, TBEV copy number is increasing commensurate to time [217]. However, whether even shorter feeding time supports transmission of the virus from tick to tick or if a tick can infect a new host when continuing interrupted feeding has not been studied. Also, voles develop a systemic TBEV infection more often compared to mice [218]. Whether shortened duration of tick feeding affects the infection kinetics is not known.

According to our results one might speculate, that even if certain rodent species may contribute to the activity of the TBEV-focus, the dominant rodent species is not the determining factor for TBEV-focus establishment. *M. glareolus* dominated in TBEV foci surveyed in the present study. Resistance of *M. glareolus* to ticks in different foci in Finland needs to be considered further: is co-feeding less important for TBEV maintenance in boreal region or do the biological determinants for non-viremic transmission differ between rodent host populations, and does the recent introduction of TBEV limit the generalization of the findings?

TABLE 1. Wild small mammals

TRAPPING SITE		TRAPPING DATES	SPECIES CAUGHT (individuals positive for TBEV in molecular or serological testing/isolate gained)							
			Myodes glareolus	*Microtus agrestis*	*Apodemus flavicollis*	*Arvicola amphibius*	*Sorex araneus*	*Sorex caecutiens*	*S.ssp*	*M.ssp*
Kokkola		11.-12.3 2008 9.-10.3. 2009*								
	Enträskholmen 63°90'N,22°87'E		8 (Se:1)	-	-	-	2	-	-	-
	Börskär 63°88'N, 22°78'E		5	-	-	-		3	-	-
	Harrbådan 63°88'N,23°09'E		19	-	-	-	2	1	-	-
	Trullevi 63°91'N, 23°07'E		39 (PCR:4,Se:5, Is:1)	-	-	-	2 (PCR:1)	-	3	
	Norra Hamnskäret 63°92'N, 22°80'E		9	-	-	-	1	-	-	-
Helsinki	Isosaari 60°10'N, 25°6'E	19.-21.2. 2008 4.-6.3.2009	-	95 (PCR:16)	-	1	13 (Se:2)	-	-	-
Varkaus (Serology NA)	62°30'N, 27°90'E	17.-18.6. 2009	7	1	2	-	2		-	**1**
Simo		24.-26.6. 2009								
	Ykskuusi 65°66'N, 24°78'E		9 (PCR:7,Se:4, Is:4)	-	-	-	-		-	-
	Maksniemi		8	-	-	-	1		-	-

	65°65'N, 24°72'E		(PCR:8)						
	Control area		-	-	-	-	-	-	-
Kuopio (NA)	Jännevirta 62°97'N, 27°85'E	25.27.8.2010	12	-	-	-	-	5	-
Kotka		8.-9.6.2011							
	Kyminlinna 60°51'N, 27°89'E		7	12	-	-	1	-	-
	Kirkonmaa 60°40'N, 27°04'E		-	2	-	-	3	-	-
	Haapasaari 60°28'N, 27°19'E		-	2	-	1	8	-	-
	Sisä-Nuokko (Hamina) 60°45'N, 27°22'E		3 (Se:2)	1	-	-	-	-	-
Sipoo (NA)		7.-8.2013	-	-	-	-	-	-	-
	Mångsholmen 60°24'N, 25°24'E		21	-	3	-	1	1	1
	Karhusaari (Helsinki) 60°25'N, 25°24'E		-	-	1	-	-	-	-

*2009 trapping only in Trullevi and Harrbådan
NA, not analyzed
Se: Positive in serological test (HI or IFA)
PCR: positive in real-time RT-PCR
S. Ssp: *Sorex* species
M. Ssp: *Myodes* or *Microtus* species

TBEV infection in host rodents (I-III)

M. glareolus and *M. agrestis* were shown to harbour TBEV-RNA, mainly in brain tissue, outside of tick-feeding season in both TBEV-Sib and TBEV-Eur foci in Finland (Tables 1 and 3). Also, in the experimental setting TBEV was shown to be highly neurotropic in *M. glareolus* and clearance of RNA was slower from brain than from other organs (Figure 9). In contrast to non-host species, the outcome is seldom detrimental in these natural host rodents. Neuronal death was rare and apoptosis absent in our study making bank voles excellent models for studies of TBEV encephalitis.

The distribution of virus antigen in *M. glareolus* differed from that reported in humans and dogs: in the first phase of infection virus antigen was found in cortex and hippocampus and later mainly in cerebellum, while in humans and dogs a wider distribution is seen [174, 175, 219], possibly providing one explanation for the more severe clinical picture in the latter species. Also, a relatively high phagocyte response was seen in *M. glareolus,* inferring an efficient clearance of the virus. In most cases inflammation was seen in histopathological examination, in particular at 8 dpi, and passed by without causing clinical symptoms of any kind. However, efficient clearance is irreconcilable with the persistence seen in our studies.

In endemic areas *M. glareolus* voles are considered to be resistant to TBEV infections due to long host-parasite evolution. Furthermore, development of persistent infections may be more likely in indigenous species than in new host species [33, 137, 220]. Cell-specific suppression of apoptosis in invertebrates and mammals, supporting TBEV maintenance, has been suggested as one possible factor [33]. In Finland TBEV has been presented recently, at least in some areas, and phylogenetic data reflects rather random geographical distribution than continuous spread along with host rodents and parasitizing ticks [53]. Host resistance to TBEV-infection due to long-term co-existence is in contradiction with the assumption that a TBEV-focus could only be established where bank voles have not yet developed immunity to ticks.

Persistence of TBEV in small mammals

In some publications the term latency is used to refer to TBEV-positive findings after several months after infection, while latency may also refer to integration of the viral genetic material into host genome. We chose to use the term persistence, because the mechanism for how the virus or components of the virus are remaining in host is unclear. If the virus is active and replicates at low levels is not known. It is also controversial, if one can claim, that the virus is persisting, if only RNA or certain viral proteins are shown [220], while no infective virus is found. In this study, we refer to persistence even if only TBEV-RNA was shown in any organ.

In the present study, we detected TBEV-RNA in *M. glareolus* and *M. agrestis* voles in winter several months after tick activity season and up to 168 days post syringe infection in bank voles (Tables 1-3).

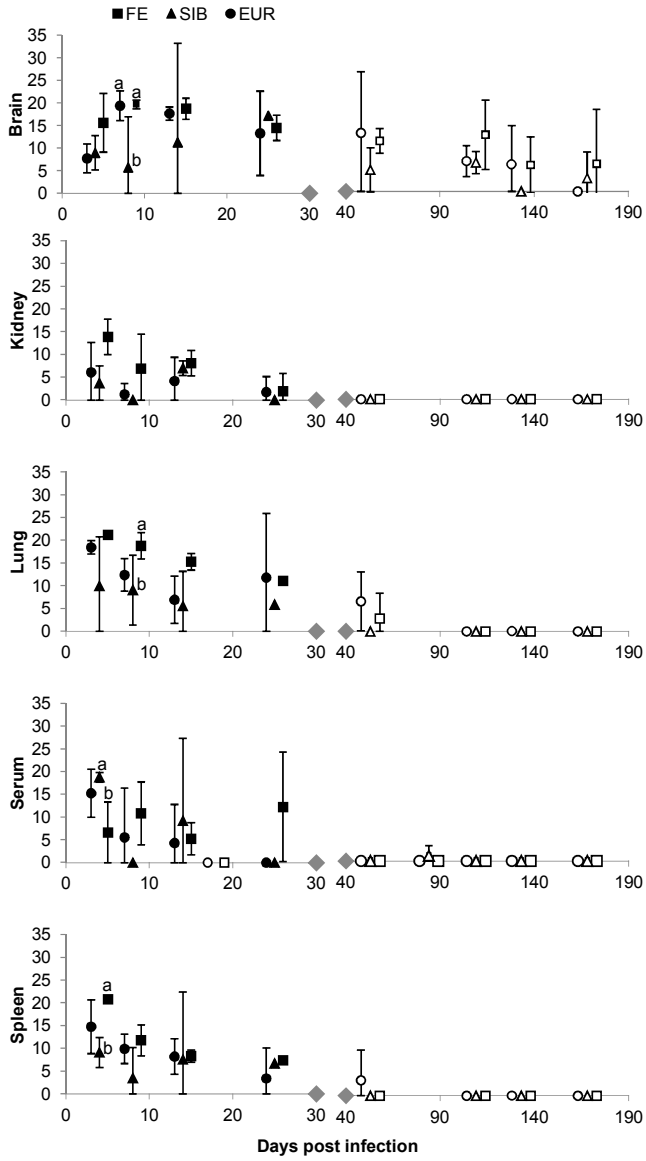

Figure 9. PCR results of the experimental infection study Relative quantities of TBEV (inverse RT-PCR cycle threshold value, mean ± 95% confidence interval) in bank vole tissues and serum during the short-term (closed symbols) and long-term experiments (open symbols). Strains that differ statistically significantly within a day post infection ($P<0.05$ in pairwise Tukey comparison) are marked with a and b. Notice the different scales on the separated parts of the x axis. EUR = European, FE = Far-Eastern, SIB = Siberian TBEV subtype. Courtesy of Liina Voutilainen.

Table 2. Results of the experimental infections of bank voles, *M. glareolus*

Dpi	Subtype	PCR	EIA	Inflammation	Antigen
			Acute infection kinetics		
4	Eur	3/3	0/3	0/3	**0/3**
	Sib	3/3	0/3	1/3; E	**0/3**
	FE	3/3	0/3	0/3	**0/3**
8	Eur	3/3	3/3	3/3; Me,Me,Me	**3/3**
	Sib	1/3	1/3	1/3; Me	**0/3**
	FE	3/3	3/3	3/3; Me,Me,E	**3/3**
14	Eur	4/4	4/4	2/4; E,E	**3/4**
	Sib	1/4	1/4	1/4; E	**1/4**
	FE	4/4*	4/4*	1/4; Me	**3/4**
25	Eur	3/3	3/3	2/3; E,E	**0/3**
	Sib	1/3	1/3	0/3	**0/1****
	FE	3/3	3/3	0/3	**NA**
			Persistence		
53	Eur	3/3	3/3	0/3	**0/3**
	Sib	2/3	2/3	0/3	**0/3**
	FE	3/3	3/3	0/3	**0/3**
109	Eur	3/3	3/3	0/3	**0/3**
	Sib	3/3	3/3	0/3	**0/3**
	FE	3/3	3/3	0/3	**0/3**
133	Eur	2/3***	3/3***	2/3;M,M***	**1/0*****
	Sib	0/3	2/3	0/3	**0/3**
	FE	2/3	3/3	0/3	**0/3**
168	Eur	0/4	4/4	1/4;M	**0/3**
	Sib	1/4	4/4	0/4	**0/3**
	FE	1/4	4/4	0/4	**0/3**

*2 euthanized at 12 dpi due to severe unspecific symptoms
** 2/3 NA
*** One of the animals scheduled to be euthanized 133 dpi died 110 dpi

Results are presented as proportion of positive results / infected individuals. The inflammation status abbreviated as following: ME=Meningoencephalitis, M=Meningitis, E=Encephalitis and NA=not analyzed/no sample. Uninfected control animals were negative in all tests.

Our results show that TBEV persists longer in brain than in other organs both in wild and in experimental settings (Fig. 9). Many flaviviruses have the ability to replicate in viscera of wild mammals without damaging them, thus entry to CNS would not be necessary for persistence. Appler et al. speculate that due to differences in immune competence, viral persistence is likely to be more efficient in CNS, than in other organs and even within CNS there may be preferences: a longer persistence of West Nile Virus (WNV) was observed in spinal cord than in brain and TBEV was found longest in the subcortex ganglia and cerebellum [166]. Rat glial cells, specifically astrocytes, have been suggested to be a reservoir for TBEV [176] and in persistent Japanese encephalitis (JE) infection, microglial cells act as viral reservoirs in nervous systems [221]. In our experimental infection study, persistent viral antigens were detected in macrophages and neutrophils, and even outside of cells 133 dpi in brain of an individual with leptomeningitis. However, this was observed

exclusively in TBEV-Eur infected individuals, while RNA was also detected in TBEV-Sib and –FE infected animals up to 168 dpi.

In general, viruses capable of causing smouldering persistent infections alter the host immune system functions by escaping recognition by host immune system or by altering lymphocyte or monocyte functions [222, 223]. Clearance of JEV is repressed in mice during pregnancy [222]. Infection of T-lymphocytes has been suggested as a mechanism for suppression of host immunity by JEV [224] and JEV could be recovered from peripheral mononuclear cells and T-lymphocytes in seroconverted children after an asymptomatic period of up to 9 months [225]. Naslednikova and colleagues suggest, that compromised T-cell functions and changes in lymphocyte relations of T-cell types and Th1 and Th2 lymphocyte subclass imbalances may contribute to the development of chronic TBEV infection in humans [160]. Other possibilities are changes in viral gene expression and posttranslational modification: in flaviviruses typically NS1, C, prM and E proteins [155, 223, 226]. Furthermore, antibody mediated enhancement described in other flaviviruses could also occur for TBEV [131]. In our persistence study TBEV-antigen was expressed at 133 dpi in macrophages, neutrophils and in addition cell-free. However, this was seen only in one individual infected with TBEV-Eur also having acute inflammation, while antigen was not detected in other individuals even if RNA was found, so the result cannot be generalized and the mechanism of TBEV persistence in bank voles cannot be readily explained based on our results.

In monkeys the duration of clearance of TBEV depended on the preceding disease course [179], but in our study RNA was detected 168 dpi without symptomatic disease in *M. glareolus*. Also, it is unlikely, that wild rodents would survive several months of winter, if persisting virus would cause significant morbidity in them. On the other hand, the highest mortality in rodents is seen late in the winter, when food runs out, so our trapping in February and March may have taken place just on the edge of the strongest selection pressure.

However, the amounts of RNA detected in brain tissue after several months post infection, both in wild and in experimental settings, were low, as well as in studies by Bakhvalova et al. [137]. Even if persistence of TBEV-RNA could be demonstrated both in wild host rodents and in the experimental study, isolation attempts were successful neither in suckling mice nor by cell passaging (Table 4). TBEV antigens have been detected in Siberia in winter in *M. rutilus*, *A. agrarius* and *S. araneus* [131, 227], and isolates of TBEV-Eur were recovered from wild *M. glareolus* and *A. flavicollis* caught in February in Slovakia [126]. Experimental infection of *Macaca rhesus* monkeys with TBEV strains Vasilchenko (Vs) [228] and Aina resulted in persistent infections where the virus could be isolated up to 783 dpi. Isolates were obtained from liver, spleen, lymph nodes and CNS, although impaired infectivity hindered the isolation success [161, 229, 230]. Pathogenicity in suckling mice and monkeys was attenuated and another study reported a persistent TBEV originating from wild *M. rutilus* but the virus could only be recovered in white mice after immunosuppression with cyclophosphan [227] (and Bakhvalova, personal communication). Persistence of other arthropod borne flaviviruses,

WNV and JEV, and reactivation by immunosuppression has been described [166, 222]. TBEV replication could be reactivated in *Mesocrietus auratus* (Syrian hamster) by treatment with stress hormones and chemical immunosuppressant drugs two years after the experimental inoculation of strain of TBEV-sib type. Earlier asymptomatic animals manifested disease and infective virus could be isolated [94, 231].

In wild rodents, reproductive hormones [94] or heavy tick infestation may cause immunosuppression [100], a phenomenon which may be accelerated by heavier tick infestation on reproductive immunosuppressed rodents. The activity of reproductive voles and active larvae and nymphs is simultaneous at least in studied foci in Siberia [94]. In the present study, we set *I. ricinus* ticks on the experimentally infected bank voles, but unfortunately only very few fed, poorly, and no conclusions can be drawn based on the results.

Whether persistence is in fact very slow clearance related to immunomodulation, or if persisting virus has potential to be reactivated and has significance for maintenance of TBEV and thereby human risk, is still to be addressed in further studies.

TABLE 3. Virus isolation experiments

Sample			Mice with paralytic symptoms	PCR positive / sequence obtained
NMRI mice				
Kokkola 2008	Bank vole 62	brain	1/13 (late)	-
Isosaari 2008	Field vole 3	brain	0/12	-
	Field vole 4	brain	1/13 (late)	
	Field vole 5	brain	3/12 (late)	
Kokkola 2009	Bank vole 10	brain	4/12	-
	Bank vole 10	lung	0/5	-
	Bank vole 17	brain+lung	3/14 (late)	-
Isosaari 2009	Field vole 13	brain	0/11	-
	Field vole 22	brain	0/10	-
Simo 2009	Bank vole 2	brain	8/11	+
	Bank vole 2	lung	1/6 (late)	-
	Bank vole 5	brain+lung	8/8	+
	Bank vole 7	brain+lung	4/8	+
	Bank vole 9	brain+lung	11/15	+
	Tick pool 38	2 ticks	12/12	+
	Tick pool 48	3 ticks	13/13	+
Vero E6 cell passage				
			CPE	**IFA or PCR pos.**
Kokkola 2008	Bank vole 62	brain	-	-
Isosaari 2008	Field vole 4	brain	-	-
Experimental	FE7	brain	-	-
Experimental	FE7	spleen	-	-

Antibody response to TBEV

Anti-TBEV antibodies were not detected in all voles trapped in winter in Isosaari and Kokkola, even if TBEV-RNA was detected (Table 1). Antibodies were shown only once in an individual with no RNA detected, indicating a high tendency of RNA to persist. The difficulties with HI method in detection of TBEV-antibodies in winter trapped rodents may have affected our first results with winter trapped rodents during 2008, but not in later examinations. Moreover, reported strain specific variations in producing HI-antibodies and recently confirmed HA-deficient variants of Baltic TBEV-Sib should be considered [131, 232].

In Simo 15 of 17 *M. glareolus* trapped (Table 1) in early summer in 2009 were positive in RT-PCR. Four of these showed high TBEV-RNA levels and these individuals exhibited exclusively seropositivity. TBEV-RNA levels of brain and lung specimens were mainly similar suggesting acute viremic infection. Lower RNA load and absence of IgG-antibodies in the rest of the animals is hard to explain. TBEV-RNA-levels were similar in brain and lungs also in these individuals, thus over winter persisted old infection is an unlikely explanation as our studies on persistence show, that persisting RNA is typically found in brain. Also, animals seemed to be young, being born in the same summer. Neither can an explanation be found in properties of the four seropositive individuals: they were all males, but size and testicle size referring to maturity varied. Our results leave open question on why some TBEV-infected individuals produce antibodies, while others do not and why antibodies are persisting in certain individuals.

Absence of antibodies in antigen and RNA positive winter trapped *M. rutilus, M. glareolus* and *A. flavicollis* have been reported previously [126, 131]. In *macaca* monkeys the spread and replication of the virus did continue even if high levels of neutralizing antibodies were detected in serum [171]. Furthermore low level and short lived recurrent antibody responses have been reported in birds during chronic JEV and WNV infections [220] and even in mice infected with JEV [233]. In humans, chronic TBEV-infections may occur without detectable antibodies and with untypical patterns of IgM/IgG responses [41, 43]. On the other hand neutralizing anti TBEV-antibodies may develop in totally asymptomatic infection [217].

Interference of the virus with lymphocytes or sub-neutralizing levels of antibodies leading to antibody dependent enhancement might be behind the impaired clearance of the virus. This is yet to be studied in natural hosts. In our experimental infection clearance was rather efficient. Seroconversion was detected in all TBEV-Eur and TBEV-FE infected individuals, but seroconversion was slower or did not happen in all TBEV-Sib infected individuals (Table 2), which might reflect special interactions of TBEV-Sib with host rodents. Notably, an individual infected with TBEV-Sib was found RNA positive in serum still at 84 dpi. In addition, IgG levels reached extreme at 84 dpi. The same individual demonstrated RNA in brain, but not in serum at 168 dpi. However, syringe infection in salt buffer may cause significant bias compared to natural infection by a tick bite [110].

Generally, life-long persistence of neutralizing anti-TBEV antibodies makes the hosts dead-ends for virus maintenance via viremia. Short-term or recurrent neutralizing antibody response would question this assumption.

Rodents as sentinels: know your rodent

Because of the low prevalence of TBEV in ticks (at least in *I. ricinus* area), extensive surveys on TBEV endemic areas using ticks as sentinels are the most laborious. Furthermore, these surveys mainly aim to assess human risk, but the prevalence of TBEV in questing ticks does not correlate with the risk of morbidity [234, 235]. Anti-TBEV-antibodies have been screened in several wild and domestic mammalian species and also several bird species have potential to serve as sentinels for TBEV [220, 236-246]. The selection of suitable sentinels for TBEV needs to be done according to the aim of the actual study: dogs often follow the tracks kept on a lead though ticks further in the forest or thicker vegetation may not attach to them. Cattle, on the other hand, are often kept in open fields or even inside nowadays, thus tick bites are rare. Wild ungulates provide a good model, but restricted geographical coverage of some species or the wide range of some, like elk, make the conclusions of the virus distribution vague. Large serological surveys on humans to determine the geographical distribution of TBE are problematic as tick bites are often got in other municipalities, than the one of residence (study IV) and previous immunizations or unnoticed natural infections may bias the results of seroprevalence.

Wild rodents are numerous, heavily infested by ticks and local, which makes them potentially good sentinels for TBEV. However, our studies show that the understanding of the local biome is crucial for selection of the right season and trapping method, and for a correct interpretation of the results. Comparison of the seroprevalence in rodents between different studies is rarely appropriate as several parameters are highly variable: In Slovenia, the IgG prevalence in *M. glareolus* was found significantly higher than in *Apodemus* mice [218], while in a long term study in Siberia prevalence was higher in *M. rutilus* than in *A. agrarius* or shrews [131]. In our study IgG was more often detected in *M. glareolus* than in *M. Agrestis* in winter, although the TBEV-subtypes were different (TBEV-Sib and TBEV-Eur respectively). In addition, rodent species differ in susceptibility, infection kinetics such as the level of viremia and the immunological response [110, 126, 153, 218], hence a good sentinel species may be different than a good species for maintenance of TBEV circulation.

According to our experimental infection study, *M. glareolus* voles develop a systemic infection prior to the virus entering the CNS. In wild rodents virus RNA was detected in brain tissue, but animals were often sero-negative. Efficient antibody response may not always develop or antibodies may not persist. Interestingly, in Simo some of the animals had antibodies, while some had less RNA in organs and antibodies were not detectable. In Kotka, the situation was the opposite – antibodies were detected, but no positive samples could be found with PCR (Table 1, Jääskeläinen A., Tonteri E. et al., manuscript in preparation).

TBEV is clearly neurotropic in *M. glareolus* and *M. Agrestis*, the main hosts for the virus in Finland in foci studied by us and others [209]. Brain was usually the only organ, where TBEV-RNA could be detected. Isolation of bank vole-derived viruses was successful from brain or brain plus lung homogenates, but not from lung alone even if the infection could be considered as recent and acute. This suggests that the brain is the best target organ for detecting TBEV-RNA in each case using rodents as sentinels for TBEV, at least in northern regions (Table 3).

TBEV-subtypes

The determinants for the maintenance of the TBEV subtypes, and their pathogenic characteristics are controversial. Due to the former iron curtain separating the eastern and western tradition of TBEV research, a considerable amount of relevant data on TBEV-Sib and TBEV-FE subtypes have been published only in Soviet journals or are still behind language barriers for many TBEV researchers.

Genetic determinants of the viruses [68, 247] and divergent interpretations of the various symptoms may have directed the deductions about the subtype-specific differences in pathogenesis and virulence [230]. It is clear, that strains within the subtypes vary greatly in their virulence according to origin of the isolation, time-point and the passage history, thus conclusions can only be drawn according to each study on a certain strain [37, 47, 200, 248]. Diverse disease course and differences in the healthcare and reporting systems in different endemic areas make comparisons difficult [188]. Also, local condensation of host genetic factors may expose to severe disease forms [181-183], which may cause bias when concluding the characteristics of TBEV strains circulating in certain areas. It has also been suggested that the length of the cold season contributes to the pathogenicity of TBE, thus diminution of the pathogenicity of the strains from east to west would be due to climatic factors [249].

Monkeys infected with TBEV-FE develop more often acute fatal encephalitis, compared to others infected with TBEV-Sib strains [68, 179]. In experimental infection studies using sheep and monkeys as models, intracerebral infection produced encephalitis in both species. RSSE (TBEV-FE) was more neurotropic and caused neuronal degeneration while CEE (TBEV-Eur) was targeted to lymphatic nodes and only 6-9 days later to brain in those animals that developed encephalitis (second phase). No neuronal damage was seen. In addition, the subtypes had different cell targets [120]. Also, different receptor preferences of TBEV-FE and TBEV–Eur have been suggested to affect the pathogenicity [231]. Experimental infections on Syrian hamsters support the suggestion that the three subtypes described by molecular analysis can be distinguished also by their pathology [33, 41]. TBEV-FE and TBEV-Sib seem to differ in their cytopathogenic characters and target organs [41].

Two subtypes, TBEV-Sib and TBEV-Eur are circulating in Finland [78, 79]. In phylogenetic analysis, the Finnish TBEV-Sib clusters with the Baltic variants

of Siberian subtypes [70]. In our study, TBEV was shown to persist equally in both endemic areas in wild small mammals. Thus we suggest, that these strains representing the two subtypes do not cause severe neuronal damage in voles since these individuals had survived until late in winter (Table 1).

In the experimental infections with strains of all known three subtypes, TBEV-FE was found in any of the studied organs significantly more often than the other subtypes. The FE-subtype (and in one case TBEV-Sib) also induced prolonged viremia compared to the two other subtypes (Fig. 9, Table 2). However, there was no evidence that any of the three subtypes would stand out concerning the pathogenic properties or target tissues.

TBEV-Eur induced leptomeningitis still at 110-168 dpi and in one of three animals the outcome was lethal. No histopathological markers of inflammation were seen in bank voles infected with the two other subtypes at this time point (Table 2). The observation cannot currently be explained. One can speculate, that TBEV-FE and TBEV-Sib are more adapted to *Myodes glareolus* than TBEV-Eur, whose ancestors diverged from the latter two about 3000 years ago [70]. However, we did not observe any significant difference between the subtypes concerning the acute infection kinetics and target organ distributions, except for the prolonged viremia in TBEV-FE infected animals. One should keep in mind, however, that our experiment represents only these three strains isolated at certain phases of circulation in ticks at each focus.

Host-adaptation and host switch

We collected small mammals and ticks in Simo at the recently emerged endemic area in Finnish Lapland. The collection was done at the very places of human infections – from the yards of the summer cottages of the patients (with their kind permission). Six virus isolates were obtained, two from ticks and four from *M. glareolus* (Table 3). The tick species at all sites in Simo was *I. persulcatus*, but the virus subtype turned out to be TBEV-Eur. In Kokkola, the nearest studied TBEV endemic area 220km south of Simo, the combination is typical: TBEV-Sib and *I. persulcatus* [78]. TBEV-Eur is found in south-western parts of the west coast, although the hosting tick species is *I. ricinus* [53, 65, 79].

The switch of tick host of TBEV is not unique. Besides reports on TBEV in non-typical tick hosts [62, 63, 126], also non-typical combinations within the traditional hosts *I. ricinus* and *I. persulcatus* have been shown, especially in the Baltics and in Western Russia [51, 250, 251]. The range of the tick species overlaps in Western Russia, Estonia, Latvia and Finland. All three TBEV subtypes co-circulate in Estonia and Latvia, although TBEV-FE is rare and its establishment unclear. The host tick populations are partly sympatric, while in Lithuania only *I. ricinus* and TBEV-Eur have been found [53, 57, 78, 79, 250-254]. An understanding of the routes of the migratory birds is necessary when prospectively viewing the possible origin of the strains [255]. Interestingly, also an understanding of history of Europe and Asia is essential. Even if TBEV-FE is considered to have evolved continuously, the mosaic pattern of distribution can be seen in former Soviet countries. In contrary to e.g.

Finland, in the study in Russia the routes of the migratory birds do not correlate with the reported pattern of virus distribution, and has been explained by anthropogenic action, the ambitious resettling of game animals [57]. To blur the picture even more, in areas where TBEV-FE and TBEV–Sib are co-circulating in Siberia, virus isolates of variants with mixed genotype of the two subtypes have been found [249].

The determinants for TBEV adaptation to host ticks and small mammals have not been studied extensively. The persistence in ticks may cause selective pressure in tick populations and alter the phenotype of the virus in cell cultures and the virulence in the vertebrate hosts. Also the replication in rodents, the most abundant host mammals, is likely shaping the virus population [37, 46, 163, 256]. The genetic determinants of another tick-borne flavivirus, Langat virus, for the replication in tick or mammalian host were located in regions in structural proteins M and E and NS3, NS4 and NS4B [37, 71]. Frey et al. located only one amino acid difference in full TBEV genomes isolated from *Ixodes* ticks and *M. glareolus*, in the NS2 protein. It has to be noted though, that both strains were passaged several times in mice prior to sequencing [71]. A rapid change in phenotype after the adaptation from ticks to mammals has been described and it has been suggested, that this is because the wildtype viruses occur in quasispecies rather than due to random mutations [256, 257]. By phylogenetic analyses of full-length genomes, E gene and NS3 formed a tree in which subtypes clustered together regardless of the host species [69, 250].

The E protein is an important determinant for host selection of the flaviviruses [38, 228, 258]. The hydrophobicity of E-protein has been suggested as an adaptation factor for TBEV to tick hosts. Khasnatinov et al. suggested, that the adaptation of the Siberian subtype strains to *I. ricinus* ticks results in an HA-deficient phenotype with enhanced virus transmission between co-feeding ticks [232]. However, the E and NS3 sequences of TBEV-Sib strains, isolated from *I. ricinus* and *I. persulcatus* were identical or highly convergent in another study [250]. In Simo, in the tick isolates and in one out of four *M. glareolus* isolates the 1208 nt stretch of the E gene and partial NS3 sequences were identical. Other *M. glareolus* derived sequences differed for 1 nt and 1 aa compared with the others. The isolates were obtained from brain tissue and RNA load was high as determined by real-time RT-PCR suggesting that the infection had been initiated earlier and virus was already replicating in host tissue rather than tick derived particles. We have not yet studied other parts of the genome or the phenotypes of the other strains.

Even if host-switches of TBEV subtypes have been reported in several studies, the continuity of the focus has been unclear or the efficiency of the virus replication in new hosts attenuated [259]. In Simo, an increasing number of human cases have been reported since 2008 (Table 4). Our results suggest that virus-host boundaries are not insurmountable when a new TBEV focus is establishing, but traditional tick-subtype combinations are rather consequences of geographical distributions and biome preferences of the host tick species.

The virulence in humans has been associated with vector species among mosquito-borne flaviviruses [23]. It would be interesting to analyse the disease course of human cases in Finland in known foci with different combinations of TBEV subtypes and hosting tick species. However, possible year-to-year variation in virus virulence and the relatively low number of cases in Finland hinder such studies.

Evidence for new endemic areas in Finland and human risk for TBE infection (I, II and IV)

All TBE cases in Finland since 1995 are available in the Finnish National Infectious Disease Register. The cases are reported by the hospital district of the treating unit. Further details on the disease course, vaccinations and recognized tick bite have been collected since 2007 by the National Institute of Health and Welfare. We surveyed the human TBE cases in Finland between 2007-2013 according to the place of infection to create a map of the geographical distribution of TBE infections (Figure 10) and to survey the development of case numbers in each TBEV focus (Table 4). The coverage of the data with a known place of infection (208 cases) compared to all cases registered during the period (233 cases) was 89,3%. Residents of Åland were not interviewed - if no other origin of infection was given, it was assumed, that the infection was obtained in Åland. In our data, 67,7% of patients with TBE diagnosis were male. The median age of male patients was 48 (range 1 to 85) and female patients 50 (range 4 to 81), while in the general population of Finland in 2013, the median ages were 39 and 43 respectively according to Statistics Finland. 49% of the patients were over 50 years old, which is comparable with central Europe rather than with Russia, where young age groups are most affected [105].

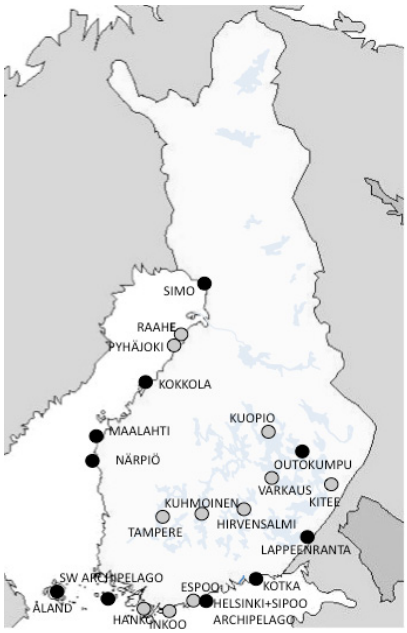

Figure 10. Human TBE cases in Finland 2007-2013, by the geographical sites of infection.

Sites with repeated human cases during the study period are marked with black dots. Sites were only sporadic cases occurred and maintenance of TBEV in area is unclear are marked with grey dots.

Data of geographically continuous areas comprising several municipalities were combined as following: Simo: Simo and Kemi; Helsinki: Helsinki and Sipoo archipelago; Lappeenranta: Lappeenranta (LPR), Imatra, Lemi, Joutseno; Kotka: Kotka and Hamina archipelagos; Kokkola: Kokkola and Kannus. South-western archipelago reaches to the south-east to Kemiö, in north we included sites until the archipelago of Uusikaupunki and in west the archipelago is limited to Åland

Table 4. Human TBE cases in Finland 2007-2013 by the site of infection

	Åland	South-western archipelago	LPR region	Simo and Kemi	Kokkola region	Kotka and Hamina	Helsinki-Sipoo region	Maalahti	Pyhäjoki	Kuopio	Outokumpu	Espoo	Hirvensalmi	Varkaus	Inkoo	Kitee	Närpiö	Tampere	Kuhmoinen	Raahe	Hanko	Other countries	All cases with known site of infection	All registered cases
2007	10	4	2	0	3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	**20**	**20**
2008	12	3	1	2	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	**21**	**23**
2009	5	7	2	2	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	**19**	**26**
2010	14	5	4	1	3	2	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	3	**35**	**44**
2011	11	13	1	1	0	2	4	2	0	0	1	0	0	0	1	0	0	0	0	0	0	5	**41**	**43**
2012	6	11	2	7	0	4	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	3	**36**	**39**
2013	9	6	4	2	1	0	0	0	3	0	1	2	0	0	0	0	0	1	1	1	1	4	**36**	**38**
All	**67**	**49**	**16**	**15**	**9**	**8**	**6**	**4**	**3**	**2**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**16**	**208**	**233**

Data on geographically continuous areas with individual foci were combined as explained in caption of figure 10.

The highly endemic areas, the south-western archipelago and Åland in the Baltic Sea have been known for decades for the high TBE incidence, even when considering the scale of the whole of Europe [5, 187]. The last countrywide survey in Finland on TBEV distribution dates back to 1960s, when anti-TBEV antibodies were screened in cattle sera throughout the country [241]. Besides Åland and the south-western archipelago, where virus was also isolated from ticks [209], positive serum samples were found in South-Eastern Finland and in the Kokkola region, where occasionally human cases have also been reported throughout the years. In Kokkola, a cluster of human cases arose in 2002 [260].

Individual TBEV infections obtained in the area between the south-western archipelago and Närpiö are also known to have occurred before our study period (personal communication, the patient), suggesting that the west coast can be considered as a potential risk area for TBE as whole. In the present study we showed, that new geographical sites of origin for human TBE infections are emerging along the west coast. The northernmost sites in Raahe, Pyhäjoki and in the Simo-Kemi region are also the northernmost known TBEV-foci in the world. High prevalence of the TBEV-Eur was detected in *I. persulcatus* ticks and in *M. glareolus* in Simo.

In addition to the coastline of Baltic Sea, cases were registered during our study period in Lappeenranta region (LPR), where TBEV was isolated already in 1973 [209], and other locations around Lake Saimaa, the biggest waterway in Finland. In 2013, individual cases were reported in Kuhmoinen, by another big waterway, Päijänne, and in the city of Tampere, which is also surrounded by big lakes. This suggests the emergence of new foci or increased awareness of TBE.

Except for Åland, that is considered overall an endemic area, the virus is found in foci in Finland, even in risk areas like the western coastline. An endemic TBEV focus lies typically by water along the routes of migratory birds and is geographically isolated and restricted; an island or a spit. Moist soil by water may favour the survival of the tick population, highly sensitive to dryness [95]. Also, the microclimatic conditions by big waters, like slower cooling in the autumn may contribute to tick abundance [261]. Geographical isolation shapes the host populations of ticks and may generate a situation where tick bites concentrate on certain rodent individuals as bites are not diluted to larger mammals [13]. An intensive co-feeding leads to efficient TBEV transmission between ticks.

Importantly, not only are ticks comfortable when isolated next to water, but also Finnish holidaymakers seek isolation in summerhouses situated by watersides, when weather is also optimal for tick questing. In the present study, even if the place of infection was the municipality of residence or the neighbouring one, TBEV was, as a rule, transmitted at the summerhouse, especially in Simo and Kokkola. Long exposure time at summerhouse increases the risk of getting bitten by an infected tick and notably the increasing number of fit pensioners, at the risk age of severe TBE-infection (at least TBEV-Eur) [188, 191] are spending longer periods at the second home. Simultaneously with the increase in TBE case numbers, the number of

summerhouses has doubled from that of 251744 in 1980 to 496208 in 2012, although during the 2000s the building of new recreational homes has stabilized. In the area of Varsinais-Suomi hospital district in South-Western Finland there are more than four summerhouses per km² [262]. In Parainen the incidence of TBE is 16,8/100 000 residences of the municipality. Still, there are more summerhouses, than permanent residences [262] and the area is also popular among boaters which is in line with observations of the present study: the majority of the cases were reported among visitors treated in other hospital districts. These contribute particularly to cases registered at the hospital district of Helsinki and Uusimaa (HUS). Even in Parainen the whole municipality cannot be considered evenly endemic. Of defined locations within Parainen, 57% of infections were obtained in Nauvo and the rest in Korppoo and Houtskär, suggesting focality, even if biological and geographical conditions would allow a continuous occurrence of TBEV. Of the areas with highest incidence, the Lappeenranta region makes an exception as there many of the TBEV foci are in the city area and infections are mainly obtained during everyday activities.

Åland is another favourite area of summer tourism and boating, although visits may be shorter than in areas, where several weeks are spend at summerhouses owned by people living in other areas. Land ownership in Åland is restricted and number of summerhouses is approximately only one tenth of that in Varsinais-Suomi [262]. TBE incidence in Åland was 99,97 (1/100 000 inhabitants) in the peak year of 2002, but has decreased after the national vaccination program provided to local inhabitants was initiated in 2006 and fluctuated in 2007-2013 between 10,93 and 39,66. The vaccination coverage is at present 70,7% [263], but according to our observations in 2007-2013 together, Åland was still the most common site of infection and most of the infections were obtained by the local residents. When evaluating the TBE cases reported to NIHW by NIHW and the representatives of the diagnostic laboratories, a few vaccine failures per year were found, some also in Åland. In a study of 533 individuals carried out in Åland, the risk for insufficient titer of neutralizing antibodies after the immunization correlated with high age, dose number and previous history of vaccination against JEV [196]. However, in general in Finland vaccine failures were found in all age groups.

The national working group on TBE-vaccinations has also recommended a vaccination program in Simo and Parainen [263], although in the latter area in south-western archipelago the high number of summerhouses and visitors complicates the allocation of the costs. However, in recent years the number of infections obtained in south-western archipelago have reached or in some years even exceeded those in Åland. In the northernmost part of the Gulf of Bothnia a considerable proportion of the patients infected in Simo are living in a neighbouring municipality, Kemi or even vice versa, when infections are obtained in Kemi. Also, infections are, at least to date, exclusively transmitted in coastal area of Baltic Sea or the big lakes. Thus, immunization should be targeted to those active in risk areas rather than all local residents of a certain municipality.

In some endemic areas, such as the Kokkola region, the case numbers are decreasing likely due to active voluntary immunizations. The conclusion is

supported by the observation in the present study where we detected anti-TBEV-antibodies and TBEV-RNA in wild rodents trapped in Kokkola and Isosaari-island in two consecutive winters (table 2) even if the human cases are currently few or absent. In addition, it has been suggested that the infectivity of the local TBEV strains may vary from year to year: virus phenotype may depend on local tick-rodent dynamics [46]. Low level of TBEV in a biting tick may not lead to clinically symptomatic disease or seroconversion in humans, and even feeding time may influence the transmission. The tick life stage may contribute to the level of infective viruses [52, 96, 217]. High incidence in young age groups reflects increased infection pressure. In Åland, where case numbers are highest, one can speculate that annual fluctuation could be seen in age distribution of the reported cases, though it is hard to conclude readily, if the difference is due to the properties of the circulating viruses without actually studying them. However, the genotype of TBEV isolated in Kumlinge, Åland seems to be stable [70]. A cyclical pattern of *I. persulcatus* population dynamics has been described in Siberia [249] and this is possibly also worth considering in Finland.

In the present study we described a new focus with continuous human cases of human infections as far north as in Simo at the 65th parallel of latitude. Three severe TBE cases were also reported in 2013 in Pyhäjoki 140km south of Simo (Fig 10), but tick species and the TBEV subtype are so far undetermined. The tick species carrying TBEV in Simo was *I. persulcatus*, which has earlier been found in Kokkola and Närpiö south of Simo at the west coast of the Baltic Sea (Fig 10) [65]. The tick species may affect the human risk and the infection time point: while for *I. persulcatus* it is typically adult females, which transmit TBEV to humans, for *I. ricinus* it is nymphs that transmit the most human infections [95]. The virus load in *I. persulcatus* ticks is revealed to be higher than in *I. ricinus* [249]. However, in Simo the TBEV-Eur virus load in *I. persulcatus* varied extensively and no such conclusions could be drawn. The severity of the infections transmitted in TBEV-Eur vs. TBEV-Sib endemic areas is yet to be evaluated. In Pyhäjoki, where tick species is probably *I. persulcatus*, all three cases reported were severe and patients needed intensive care. However, the factors generating such a pathogenic strain in Pyhäjoki 2013 need to be evaluated in further studies.

New TBE foci are also emerging in the inland regions of Finland (Fig.10, Table 4). The TBE incidence on the whole has been increasing since 1995, despite the immunization program initiated in Åland in 2006. However, the case numbers have been fluctuating. Incidence peaked in early 2000, then mainly due to the high incidence in Åland. Since 2010 there seems to be another peak, with weight shifted to other parts of the country. Thus it can be concluded that excluding the bias caused by immunizations the overall case TBE incidence is increasing. Simultaneously reported case numbers of another tick borne disease, Lyme borreliosis have increased linearly and in 20 years the case numbers have quadrupled [264].

Climate change is often invoked as a straightforward explanation for change of the incidences of vector-borne diseases, including TBEV. Recently, headlines of the tabloids have been disseminating news like: “Consequence of the climate warming: Encephalitis (brain fever) ticks are spreading to whole

Finland" (Iltalehti 5th May 2014) and media often refers to *I.persulcatus* as "killer tick". The milder winters and lengthening questing season of ticks together with increased rainfall favour the increase in tick abundance and ticks are expanding to northern latitudes and higher altitudes [60, 265]. A warmer climate could be assumed to accelerate the lifestage-per-year -cycle in ticks, but this may not only be dependent on the temperature, as in temperatures allowing two life stages during the activity season, ticks still fed and moult only once per year [127]. The rise of TBE in Europe does coincide with climate warming. Also, the changing seasonality leading to the coincident activity of ticks and the competent rodent host species and individuals may benefit the maintenance of TBEV [266, 267]. At the same time, warmer summers and periods of dryness may eradicate ticks and TBEV in some traditional endemic areas in southern Central Europe [268]. On the other hand the sudden increase in TBE case numbers in Baltics in 1990s cannot be explained by climatic factors only and the North Atlantic Oscillation has no significant effect on neither the TBE cases nor the host population dynamics in Sweden. The summer temperatures in general had some positive correlation with human cases reported, but the only significant factor revealed in the model was the prey-predator dynamics of European hare and red fox [269, 270].

In Finland, follow-up data on tick abundance is only available from the island Seili in the south-western archipelago. From 2002 to 2012 the number of adult *I. ricinus* observed on the island have increased 25-fold (Ritva Penttinen, University of Turku, personal communication). The tick distribution has last been studied systematically in Finland over 50 years ago, when the northern boundary for *I. ricinus* ticks was drawn approximately to a line between Joensuu and Kokkola [241]. In Sweden, *I. ricinus* is found in areas with ≥ 175 days of annual snow cover and the species is continuously present if the period of snow cover is shorter than 125 days. Even if snow shields the ticks and their host rodents, it shortens the vegetation period during which ticks are active [54]. The vegetation period, i.e. days with mean temperatures >+5°C, has become longer in Sweden and the northwards expansion of *I. ricinus* and increase of TBE cases correlates with a warming climate together with unusually warm conditions in recent years [95, 271]. However, it has to be noted, that the areas with high TBE case numbers in Sweden as well as Baltic states lie south of the southernmost parts of Finland, probably explaining partly the 5 to 6 times higher case numbers in Sweden and Estonia compared to Finland in recent years. While in Sweden established populations of only *I. ricinus* ticks are found (Thomas Jaenson, personal communication), in Finland the limit for tick distribution in sense of the vegetation period is indeed not set to that demanded by *I. ricinus*. In the Archangel region in Western Siberia *I. persulcatus* expands northwards and the increase in TBE incidence correlates with increased temperatures as well. Although also in the Archangel, the region, where *I. persulcatus* is considered established, lies South of Simo in latitude [58].

Importantly, even if the tick abundance increases and consequently prevalence of tick-borne diseases like Lyme borreliosis distributed congruently with the ticks increases commensurately, the maintenance factors for TBEV are complex and dependent on seasonal climatic patterns. Moisture

is the most important single factor contributing to ticks questing, although too moist conditions later in summer may decrease the questing activity [261]. Satellite mapping on the expansion of TBEV foci in Europe based on optimal co-feeding conditions for different life stages of *I. ricinus* has predicted the new foci found in Southern Finland fairly precisely [268], thus ahead of prediction. However, the model seems not predict the emerging foci in the regions where vector species is *I. persulcatus*.

In our data covering the years 2007-2013, human TBE cases were reported earliest in April 20th and up to November 9th in Åland while in 1988-2005 the seasonal distribution extended from May until December [190]. In the latter study the majority of cases were registered mainly from July to October, while in our recent survey cases were registered mainly from June to September, thus no major shift in seasonality was seen. In the Lappeenranta region, most cases were reported in August and September. Notably, the case numbers in autumn did increase in recent peak years 2010, 2011 and 2013. This is possibly due to long warm falls and autumnal tick activity, but also to the prolonged season of outdoor activities wearing less protective clothing. The summer holiday season is coincident with the concentrated midsummer TBE-occurrence. However, the warm autumns may extend the season of visits to the summerhouses. Human infections in *I. persulcatus* areas were obtained mainly in early summer, while cases within the *I. ricinus* range occurred also during the autumn. This may reflect the different seasonality of the two tick species. Also, *I. persulcatus* is endemic in northern regions, where the feeding season is simply shorter.

Fluctuations in rodent population densities have an effect on the tick abundance, but also the choice of the feeding host of the tics and thereby, possibly, the efficiency of co-feeding [95]. Our study showed that in the studied TBEV foci in Finland, the virus is maintained in a cycle of ticks and voles instead of *Apodemus* mice. Rodent cycles have dampened throughout Europe during the last two decades, which has been concluded to be due to poorer conditions for winter survival and in the end to climate change [272]. However, in Finland two very strong vole peaks have been seen during the recent years and the peaks have only dampened in Northern and Eastern Finland, while in Southern and Western Finland the cycles have strengthen and are likely to get longer [273]. The vole survival is affected rather than by conditions in cold season, by the increasing food resources available, when vegetation periods are lengthening. Interestingly, the voles benefit from long warm autumns and rainy summers [273]. Mean fall temperatures have risen in Finland in recent years according to the Finnish meteorological institute. Moreover, effect of the food resources and climatic conditions is different between *Myodes* and *Microtus: M.glareolus* voles are more generalists in food preferences. On the other hand shelter of the snow cover is peculiarly important for them [128]. The virulence of TBEV strains and thereby risk for human infections has been show to correlate with hosting vole species *Clethrionomys rufocanus* (grey-sided vole) vs. *Microtus arvalis* (common vole), and this is probably due to feeding preferences of *I. persulcatus* and competing tick species [33].

Besides the climate change, human behaviour such as that revolved around

forestry, hunters pursuits and politics on carnivore control shape the host populations of ticks including deer, middle sized mammals and rodents, and in the end, the maintenance of TBEV foci and the human risk for TBE [105, 152, 270].

In Sweden, the population of roe deer, *Capreolus capreolus,* that is considered the key host for ticks, has benefited from clear cuts and winter-feeding provided by the hunters. In addition, loss of the main predator, red fox, due to an epidemic of sarcoptic mange caused an increase of roe deer numbers and a widened distribution, which seems to be the main cause for dispersion of *I. ricinus*. Further recovery of predator population led to dampening of the roe deer population and host change of ticks. Tick-to-tick transmissions of TBEV increased, when they now fed on rodents and consequently, for its part, this let to a dramatic increase in human TBE cases [95].

The dispersal of roe deer has also been rapid in Finland since the 1990s as evaluated by the hunting frequency. The species is found by big waters, areas also favourable for TBEV maintenance. Roe deer was endemic in Finland in 16th century, but the species disappeared in 18th century due to a period of severe winters. Natural re-dispersion routes of the species came from south-east from Russia and from the Tornio-river valley in southern Lapland. Rare relocations of roe deer from abroad to Southern Finland are known to have taken place in early 19th century. To date the roe deer is the most numerous in the south-western parts of Finland including Åland, where the increase of the number of roe deer has been rapid since 1957. The increase of the population in Finland is mainly the consequence of intensive relocation of game animals in latter half of 20th century [274]. Also, the climatic conditions are optimal for roe deer in the south-western area, although small populations are found all the way to central Lapland. Snow cover is one of the most limiting factors of roe deer dispersal in Finland, thus the species may be one of the benefitting species when winters get milder. However, predator abundance is also increasing in some locations (personal communication with Juho Matala, The Finnish Forest Research Institute).

The population of another cervid species, the locally important game animal white-tailed deer *Odocoileus virginianus borealis* is also found in South-western Finland, thus not in Åland. Since 2008 hunting permits have been given also in Central Finland and Eastern Finland (Savo) indicating dispersal of the species. However, the increase of number of the white-tailed deer is rather due to the increase in local abundance rather than efficient dispersal [275]. Besides hunting, snow cover, the predators, especially strongly grown population of lynx and probably competition with elk, *Alces alces* limits the population growth (personal communication with Juho Matala, The Finnish Forest Research). Elk is the most important game animal in Finland. The species is found in the whole country except for the northern fells. We screened 1384 serum samples of wild ungulates and found several seropositive elks (approximately 1,5% of all studied animals), whose geographical shooting site correlated considerably well with known sites of human TBEV infections (Tonteri, Jokelainen et al., manuscript in preparation), suggesting that elk may, considering the wide home range, serve as sentinel for human risk.

Interestingly, roe deer was not introduced in Åland yet at the time of the first reported TBE-cases (Kumlinge disease) [190, 274]. Potential host species for ticks at the time TBEV establishing in the area may have been small and middle-sized mammals and even elk. However, no systemic analysis on hosts for ticks and TBEV or associated climate factors has been performed in Finland.

Along with the new TBE cases reported raises the awareness of the disease and the infection risk factors. On the other hand, the awareness leads to increased protection at least in some cohorts, although not necessarily in those at highest risk. Subclinical cases of TBE are common and anti TBEV-antibodies may develop without symptomatic disease [190, 217]. We wanted to study, if the increased incidence is not only a consequence of the climatic and ecological factors and changes in human behavior, but also of generally raised awareness and the increased clinical alertness. Indeed, the number of patients with a serum sample sent for serological studies because of suspicion of acute TBE to the two diagnostic laboratories increased from that of 563 in 2007 to altogether 1010 in 2011, 956 in 2012 and 1154 in 2013.

In Northern Finland the laboratory testing of TBE was rare or tests were not ordered at all in the early years of our survey, while after the cases reported in 2008 in Simo the awareness has risen. In 2012, the case number peaked in Simo to 7 and so did peak the number of suspected TBE cases. Also the hospital district of Lapland has only recently started to send clinical samples to diagnostic laboratories for TBE diagnosis. However, the expansion of TBEV to as far north as southern Lapland may not be as recent as concluded in public. Positive cow sample was detected already in 1960s in Tervola, the municipality next to Simo (unpublished, personal communication of Olli Vapalahti/Markus Brummer-Korvenkontio) suggesting that ticks and TBEV may have been present in southern Lapland already then.

On the other hand, while 20% and 14% of all patients with laboratory examination for TBEV IgM antibodies were positive for the virus in Länsi-Pohja hospital district (Simo-Kemi) and Åland, respectively, alertness in some hospital districts was overemphasized. Strikingly, of 721 samples sent from North Savo hospital district (Kuopio area) only 0,7% were positive for TBE. A strong increase in numbers of clinical samples sent for TBE testing was seen since 2008, when the first human case was reported in the area. However, only two subsequent cases with North Savo as a place of infection have been reported. Other hospital districts with elevated clinical alertness are mainly located at the west coast, which was expected. In total in Finland 5,2% of all patients tested for anti-TBEV-antibodies in one of the two diagnostic laboratories were diagnosed to have an acute TBE.

In addition we screened retrospectively samples of patients with a neurological infection with unknown aetiology during the tick-feeding season in six different years to survey if TBE is underdiagnosed in this patient group and if the undiagnosed cases are concentrated in a certain areas. All samples were kindly provided by HUSLAB. Of the 1957 studied clinical serum samples, altogether 5, representing different individual patients were both anti-TBEV IgG and IgM positives and had no previous TBE diagnosis. No geographical

correlation was found. Thus, we conclude that TBE is not significantly underdiagnosed in this patient group.

All acute positive TBE cases diagnosed are reported to the Finnish National Infectious Disease Register and are annually re-evaluated by specialists of National Institute of Health and Welfare and diagnostics laboratories. Only cases fulfilling criteria for an acute TBE infection (suitable anamnestic and clinical data, no known exposure to other flaviviruses together with positive IgM in serum or cerebrospinal fluid, and TBEV-IgG positive hemagglutination titer >10) are left in the register. Without the re-evaluation the case numbers are, incorrectly, 20-30% higher including e.g. cases with unspecific IgM reactivity without demonstration of TBEV IgG or HI antibodies. The delay in correcting the data may raise unnecessary public attention in certain areas.

Concluding remarks

In studies included in this thesis we targeted both maintenance factors of TBEV in Finland, a country lying in the boreal region at the northernmost range endemic for TBEV, and surveyed the geographical distribution of all human TBE cases during years 2007-2013. In addition, we surveyed the determinants for development of TBE incidence.

The main results providing new information about the study subject were:

1: The main rodent host species in studied foci in Finland are *Myodes glareolus* (bank vole) and *Microtus agrestis* (field vole).
2: TBEV-RNA could be shown in wild rodents both in absence and presence of antibodies.
3: TBEV-RNA was detected in wild rodents in winter, several months after tick-feeding season and it persisted up to 168 days post infection also in an experimental setting.
4: TBEV was highly neurotropic in voles both in wild and in an experimental infection study, thus we suggest, that brain is the best target organ for detecting TBEV in voles.
5: *Myodes glareolus* provides an excellent and resistant model for studies on TBE - even if acute encephalitis was confirmed by histopathological examination, no significant clinical symptoms could be seen.
6: New geographical places with confirmed patient cases are emerging in Finland. The incidence is increasing and shifting from Åland, where a national vaccination program is running, to the south-western archipelago and to mainland of Finland. In some areas, however, this may be due to the increased clinical alertness rather than or in addition to the changes in climate, ecological factors or human behaviour.
7: Geographical places of human infections were visited for further analysis. In Simo, in Southern Lapland, TBEV was circulating both in *Myodes glareolus* and in ticks. This is so far the northernmost established TBEV focus known in the world. Furthermore the hosting tick species was found to be *I. persulcatus*, but unexpectedly the TBEV subtype was TBEV-Eur. We suggest, that in the mixing zone of the two main host tick species and the virus subtypes the tick species in an area does not restrict the formation of a TBEV focus regardless of the presented virus subtype.

The study raises several subjects for future studies. The factors enabling the persistence of TBEV in *Myodes glareolus* are still unclear. Clarifying the genetic, molecular and immunological mechanisms behind the persistence might elucidate the mechanism of recurrent human TBEV-infections. Also, the mechanism of entry of TBEV to CNS is still unclear. We proved that *Myodes glareolus* provide a useful model for studies of encephalitis caused by TBEV.

Anti TBEV-antibodies were not always found in wild rodents even if TBEV-RNA could be detected. The factors behind the phenomenon and the ecological significance of recurrent or fading antibody response remain to be studied.

The efficiency of non-viremic transmission in *Myodes glareolus* should be re-estimated as studied TBEV foci in Finland are mainly maintained in a cycle of ticks and *Myodes glareolus*. Furthermore the significance of persistence should be evaluated by studies on reactivation of virus in rodents infected by a tick bite (including tick saliva) and by follow-up studies of wild rodents.

Our findings in Simo pose a question: which are the genetic determinants for the host selection of each of the three TBEV-subtypes and how does the change of the virus-carrier tick species affect the phenotype of the virus.

Finland provides an excellent opportunity to study, not only the emergence of TBE and the associated ecological and climatic factors, but also differences of the human cases caused by TBEV-Eur compared to TBEV-Sib subtypes: The reporting system and treatment are uniform in Finland, while comparing patient cases in different countries with TBEV-Eur or TBEV-Sib as endemic subtypes is often not reasonable. Our studies at each geographical site of the infection provide information of the tick species and subtype of the causative agent. Furthermore, the present study provides data on clinical alertness in each hospital district thus defining the speculations on reporting of mild/severe cases in certain areas.

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